FORM PTO-1390 DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE (REV 1-98)		ATTORNEY'S DOCKET NO. 4070.000300
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 10/088129 UNKNOWN
INTERNATIONAL APPLICATION NO. PCT/NZ00/00179	INTERNATIONAL FILING DATE September 14, 2000	PRIORITY DATE CLAIMED September 14, 1999
TITLE OF INVENTION Nuclear Transfer With Selected Donor Cells		
APPLICANT(S) FOR DO/EO/US David WELLS		

Applicants herewith submit to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau); PCT cover page; 46 pages of text, Figures 1-6 on 3 sheets, and 2 pages of Search Report.
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 16 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included and the fee in incorporated within the attached check.
13. ☒ A FIRST preliminary amendment (with Exhibits A, B and C); please calculate the filing fee based upon the claims in this amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ Power of attorney and/or address letter.
16. ☒ Other items or information: 1. International Search Report; 2. International Preliminary Examination Report (IPER); and 3. Postcard

CERTIFICATE OF EXPRESS MAILING

NUMBER EL 504118295US

DATE OF DEPOSIT: March 14, 2002

I hereby certify that this paper or fee is being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to: Assistant Commissioner for Patents, Washington, DC 20231.

Rhonda Fairchild

Rhonda Fairchild

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
UNKNOWN 10/088129	PCT/NZ00/00179	4070.000300

17. ☒ The following fees are submitted:

Basic National Fee (37 CFR 1.492(a)(1)-(5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1,000.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $860.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $760.00

international preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $690.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) ..... $ 100.00

CALCULATIONS
PTO USE ONLY

ENTER APPROPRIATE BASIC FEE AMOUNT =				$ 1,000.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				$ 130.00	
Claims	Number Filed	Number Extra	Rate		
Total Claims	53 - 20 =	33	x $ 18.00	$ 594.00	
Independent Claims	16 - 3 =	13	x $ 80.00	$ 1,040.00	
Multiple dependent claim(s) (if applicable)			+ $260.00	$ -0-	
TOTAL OF ABOVE CALCULATIONS =				$ 2,764.00	
Reduction by ½ for filing by small entity, if applicable. Not applicable.				$ .00	
SUBTOTAL =				$ 2,764.00	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$ -0-	
TOTAL NATIONAL FEE =				$ 2,764.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property) +				$ -0-	
TOTAL FEES ENCLOSED =				$ 2,764.00	
				Amount to be refunded:	$ .00
				Charged	$ .00

a. ☒ A check in the amount of $2,764.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-0786/4070.000300. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
Shelley P.M. Fussey
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Date: March 14, 2002

SIGNATURE
Shelley P.M. Fussey
NAME:
39,458
REGISTRATION NUMBER:

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: WELLS, David		§	
		§	Group Art Unit: Unknown
Serial No.: Unknown PCT/NZ00/00179		§	
		§	Examiner: Unknown
Filed:	March 14, 2002	§	
Intl. Filing date:	September 14, 2000	§	Atty. Dkt.: 4070.000300
Priority date:	September 14, 1999	§	
For: Nuclear Transfer With Selected Donor Cells		§	

EXPRESS MAILING LABEL
37 C.F.R. § 1.10

I hereby certify that this paper is being deposited with the U.S. Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" (Number EL504118295US) service on the date indicated and is addressed to Assistant Commissioner for Patents, Washington, D.C. 20231 on the date below:

March 14, 2002
Date

Rhonda Fairchild

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
BOX PCT
Washington, D.C. 20231

Sir:

The present document is a Preliminary Amendment filed in conjunction with the nationalization of International Patent Application PCT/NZ00/00179. After entry into the U.S. national stage, and assurance of a U.S. filing date, entry of the following amendments is respectfully requested. Any omitted fees are authorized to be deducted from Williams, Morgan & Amerson Deposit Account No. 50-0786/4070.000300.

AMENDMENT

In the Specification:

Prior to page 1, where the text of the application begins, please delete the double-sided cover page from the PCT stage if necessary.

At page 1, after the title, please delete the single paragraph at lines 4-7 and replace the deleted single paragraph with the following two paragraph replacement section with a sub-heading:

The present application is a nationalization of International Patent Application PCT/NZ00/00179, filed September 14, 2000, which claims priority to New Zealand priority Application 337792, filed September 14, 1999.

Field of the Invention

The present invention concerns a novel method of nuclear transfer, specifically, although by no means exclusively for use in cloning technologies for the production of mammalian embryos, fetuses and offspring, including genetically engineered or transgenic mammalian embryos, fetuses and offspring.

After page 46, please start another page (47), and insert the following heading and the following text of the Abstract, as taken from the cover page of the PCT application:

ABSTRACT

The present invention provides a method of nuclear transfer by selecting and segregating G1 cells from a donor cell population. This method is advantageous over the prior art as it provides certainty as to the stage of the cell cycle which the donor nuclei are in and allows for the production of cloned transgenic or non-transgenic embryonic cells, reconstituted embryos and whole animals for agricultural, pharmaceutical, nutraceutical and biomedical applications.

At the appropriate pages, prior to the text on each page, please delete the header that reads "WO 01/19182 PCT/NZ00/00179" if necessary.

In the Claims:

After entry into the U.S. national stage, and assurance of a U.S. filing date, please revise the claims from the enclosed PCT application as follows.

Please cancel claims 47 and 48 without prejudice and without disclaimer.

Please amend pending claims 3-6, 9, 10, 12, 14, 16-18, 20-24, 26, 27, 30, 32-34, 36-40, 43-45, 49 and 51-55 without prejudice and without disclaimer, so that the rewritten claims read as follows:

3. (Amended) A method as claimed in claim 1, wherein said donor cell population is non-proliferating and has been synchronised at any point in the G1 stage of the cell cycle.

4. (Amended) A method as claimed in claim 1, wherein said G1 cell is segregated at an early G1 phase.

5. (Amended) A method as claimed in claim 1, wherein the donor cell population is non-proliferating and comprises senescent cells.

6. (Amended) A method as claimed in claim 1, wherein said donor cell population is derived from either embryo, fetal, juvenile or adult cells isolated from an animal *in vivo* or from a cell culture *in vitro*.

9. (Amended) A method as claimed in claim 1, wherein the donor cells are adult or fetal fibroblasts or follicular cells.

10. (Amended) A method as claimed in claim 1, wherein said donor cells comprise modified cells.

12. (Amended) A method as claimed in claim 1, wherein the recipient cell comprises an enucleated oocyte.

14. (Amended) A method as claimed in claim 1, wherein the recipient cell comprises an enucleated stem cell or a clump of enucleated stem cells fused together.

16. (Amended) A method of producing cloned animal embryos which comprises transferring a segregated donor nucleus in the G1 stage of the cell cycle into an enucleated recipient cell.

17. (Amended) A method as claimed in claim 16, wherein the donor nuclei are genetically altered to produce cloned embryos having desirable genetic traits.

18. (Amended) A method as claimed in claim 16, when used to produce an animal species of cloned embryo selected from the group comprising birds, amphibia, fish and mammals.

20. (Amended) A reconstituted non-human animal embryo prepared by the method claimed in claim 16.

21. (Amended) A reconstituted non-human animal embryo as claimed in claim 20, comprising a transgenic embryo.

22. (Amended) A reconstituted non-human animal embryo as claimed in claim 20 re-cloned to further increase embryo numbers or which undergoes serial nuclear transfer to aid nuclear reprogramming and/or development.

23. (Amended) A reconstituted non-human animal embryo as claimed in claim 20, comprising a species of mammal selected from the group comprising primates including humans, rodents, rabbits, cats, dogs, horses, cattle, sheep, deer, goats and pigs.

24. (Amended) A method of cloning a non-human animal comprising the steps: (1) producing a cloned non-human animal embryo according to the method of claim 16; (2) allowing a non-human animal to develop to term from the embryo; and (3) optionally breeding from the non-human animal so formed either by conventional methods or by further cloning.

26. (Amended) A method as claimed in claim 24, wherein said cloned non-human animal is a transgenic non-human animal having a desirable genetic trait.

27. (Amended) A method as claimed in claim 26, wherein said transgenic non-human animal is a transgenic bovine or ovine.

30. (Amended) A cloned non-human animal as claimed in claim 28, comprising a transgenic non-human animal having a desirable genetic trait.

32. (Amended) A cloned non-human transgenic animal as claimed in claim 30, wherein the desirable genetic trait is selected from the insertion, deletion, or modification of a gene or genes enabling the production of pharmaceutical proteins in milk, blood or urine; production of nutraceutical products in milk or meat; production of beneficial agricultural traits to improve the quality of milk, meat and fibre production; improve resistance to pests and diseases; production of industrial proteins in milk; xenotransplantation; and for the generation of transgenic animals as models for human disease.

33. (Amended) Offspring and descendants of the cloned non-human animal as claimed in claim 28.

34. (Amended) A method of producing an embryonic cell line comprising the steps a) selecting and segregating G1 cells from a proliferating population of donor cells or from a synchronised population of G1 cells or from a population of senescent cells, and transforming a nucleus from such a segregated cell into an enucleated recipient cell; b) growing to blastocyst stage; c) recovering embryonic cells; and d) establishing an immortalised cell line *in vitro*.

36. (Amended) A method as claimed in claim 34, wherein said donor cells are human cells.

37. (Amended) A method as claimed in claim 34, wherein both donor and recipient cells are human cells.

38. (Amended) A method as claimed in claim 34, wherein the donor cells are adult or fetal cells selected from any karyotypically normal cell type and the recipient cells are selected from any cell type capable of reprogramming gene expression.

39. (Amended) An embryonic cell line produced by the method of claim 34.

40. (Amended) A human embryonic stem cell line produced by the method of claim 35, useful in therapeutic applications.

43. (Amended) A method as claimed in claim 41, wherein both donor and recipient cells are human cells.

44. (Amended) A method as claimed in claim 41, wherein the donor cells are adult or fetal cells selected from any karyotypically normal cell type and the recipient cells are selected from any cell type capable of reprogramming gene expression.

45. (Amended) Embryonic stem cells produced by the method of claim 41.

49. (Amended) A method of therapeutic cloning, wherein embryonic stem cells are produced according to claim 35 from a donor cell derived from a subject, and cultured to produce

specialised cells or tissue for transplantation in said subject or in another subject in need of such treatment.

51. (Amended) A method of treating a disease, disorder or injury which may be treated by transplantation of specialised cells or tissue, comprising administering to a patient in need thereof a therapeutically effective amount of specialised cells or tissue produced according to the method of claim 49.

52. (Amended) A method as claimed in claim 49, wherein said disease, disorder or injury is selected from various neurological disorders (*eg* Parkinson's disease), diabetes, heart disease, muscular dystrophy, various hereditary diseases, specific cancers (*eg* leukemia), spinal cord injury, burns and other afflictions.

53. (Amended) A method of drug discovery or toxicology testing of drugs using *in vitro* differentiated human embryonic stem cells produced by the methods of claim 41.

54. (Amended) A method of xenotransplantation, wherein cells, tissues and organs are isolated from the non-human cloned animal of claim 28, and used for transplantation in a human patient in need thereof.

55. (Amended) A method of gene therapy, wherein cells, tissues and organs comprise a transgene and are isolated for the non-human cloned animal of claim 30.

REMARKS

I. Nationalization

This application represents the U.S. national stage of International Patent Application PCT/NZ00/00179, filed September 14, 2000, which claims priority to New Zealand priority Application 337792, filed September 14, 1999.

As the text of the International Application was transmitted by the International Bureau, an additional copy is not required to satisfy 35 U.S.C. § 371(c)(2). Nonetheless, for the Examiner's convenience, a copy of international application PCT/AU99/00813 is enclosed in the form of the published PCT Application WO 00/18790.

Should formal amendments to the specification be necessary to conform to U.S. practice, Applicants seek to introduce such amendments into the present specification by, *e.g.*, deleting the PCT cover page, providing the Abstract as a separate page, and deleting the PCT header.

Priority is also properly claimed by an amendment at page 1.

Amendments were made to the application during PCT examination, both to the specification and claims. However, none of the amendments to the specification or claims during submitted PCT examination should be entered upon entry into the U.S. national stage. Therefore, the original, unamended PCT application forms the basis for the amendments introduced herein.

II. National Stage Claims

After according a U.S. filing date, and **before** calculating the filing fee, entry of the foregoing claim amendments is respectfully requested.

The claims do not represent the claims at the IPER stage, but the text of the claims when the PCT application was filed. The original PCT claims therefore form the basis for the present

claim amendments, which are of a procedural nature only, revising the claims to better accord with U.S. practice.

The revised claims are fully supported by the specification and claims of the international application and do not in any way constitute new matter.

III. Status of the Claims

The PCT application was filed with claims 1-55, which were pending prior to the present amendment. PCT examination indicated each of claims 1-56 to have unity of invention, which should be noted upon entry into the into the U.S. national stage.

Presently, claims 47 and 48 have been canceled without prejudice and disclaimer, as not being in accordance with U.S. practice. Claims 3-6, 9, 10, 12, 14, 16-18, 20-24, 26, 27, 30, 32-34, 36-40, 43-45, 49 and 51-55 have been amended without prejudice and disclaimer, to better accord with U.S. practice, *e.g.*, to remove multiple dependencies and to make clerical changes. No claims have been added. Claims 1-46 and 49-55 are therefore in the case.

IV. Support for the Claims

Aside from removing the multiple dependencies throughout, and introducing minor changes, current claims 1-46 and 49-55 represent those of the PCT application as filed, essentially in unamended form.

Most of the changes to the revised claims simply remove the multiple dependencies, and such changes are clearly supported by each claim itself.

In addition, claim 17 and claim 34 have been revised to delete the phrases "using methods well known in the art" and "by methods known in the art", respectively, as being redundant in U.S. claim practice.

Claims 20-23 have been revised to add the term "non-human", so that the claims recite "non-human animal embryo".

It will therefore be understood that no new matter is encompassed by any of the present amendments.

V. Compliance with 37 C.F.R. § 1.121

Copies of the pending claims are attached hereto as **Exhibit A** and **Exhibit B**. In accordance with 37 C.F.R. § 1.121, the claims have been labeled as "(Amended"), where appropriate. **Exhibit A** provides a clean copy of the pending claims, whereas **Exhibit B** shows the changes with brackets and underlining.

The proper claim for priority has been timely introduced into the specification by amendment of the opening paragraph at page 1. An Abstract is also introduced into the specification by amendment as a separate page.

The amendments to the specification have been made as "replacement paragraphs" in accordance with 37 C.F.R. § 1.121. This is proper as the amendments include the reference, replacement paragraph in clean form and another version of the replacement paragraph separate from the amendment marked up to show all changes (**Exhibit C**).

VI. Fees and Formalities

The national filing fee and claim fees are included herewith. The fees have been calculated **after** the present changes to remove the multiple dependencies throughout the claims. Any omitted fees should be deducted from Williams, Morgan & Amerson Deposit Account No. 50-0786/4070.000300.

Applicants are believed to be required to pay large entity fees, but reserve the right to request a refund should this prove to be in error.

Should the Office have any questions or comments, a telephone call to the undersigned Applicant's representative is earnestly solicited.

Respectfully submitted,

Shelley P.M. Fussey
Reg. No. 39,458
Agent for Applicant

WILLIAMS, MORGAN & AMERSON, P.C.
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Date: March 14, 2002

EXHIBIT A
NATIONAL STAGE CLAIMS

1. A method of nuclear transfer, comprising selecting and segregating G1 cells from a proliferating or non-proliferating population of donor cells and transferring a nucleus from such a segregated G1 cell into an enucleated recipient cell.

2. A method as claimed in claim 1, wherein the donor cell population is at one or more known or unknown stages of the cell cycle.

3. (Amended) A method as claimed in claim 1, wherein said donor cell population is non-proliferating and has been synchronised at any point in the G1 stage of the cell cycle.

4. (Amended) A method as claimed in claim 1, wherein said G1 cell is segregated at an early G1 phase.

5. (Amended) A method as claimed in claim 1, wherein the donor cell population is non-proliferating and comprises senescent cells.

6. (Amended) A method as claimed in claim 1, wherein said donor cell population is derived from either embryo, fetal, juvenile or adult cells isolated from an animal *in vivo* or from a cell culture *in vitro*.

7. A method as claimed in claim 6, wherein said donor cell population comprises any diploid karyotypically normal cell capable of being stimulated to enter the cell cycle and proliferate.

8. A method as claimed in claim 7, wherein said donor cell population is of an undifferentiated cellular state or are at any degree of differentiation or quiescence or senescence.

9. (Amended) A method as claimed in claim 1, wherein the donor cells are adult or fetal fibroblasts or follicular cells.

10. (Amended) A method as claimed in claim 1, wherein said donor cells comprise modified cells.

11. A method as claimed in claim 10 wherein said donor cells comprise transgenic cells.

12. (Amended) A method as claimed in claim 1, wherein the recipient cell comprises an enucleated oocyte.

13. A method as claimed in claim 12, wherein the enucleated oocyte is obtained from a species corresponding in origin to the donor nuclei.

14. (Amended) A method as claimed in claim 1, wherein the recipient cell comprises an enucleated stem cell or a clump of enucleated stem cells fused together.

15. A method as claimed in claim 14, wherein the stem cells are embryonic stem cells isolated from a growing embryo or form an established cell line in culture.

16. (Amended) A method of producing cloned animal embryos which comprises transferring a segregated donor nucleus in the G1 stage of the cell cycle into an enucleated recipient cell.

17. (Amended) A method as claimed in claim 16, wherein the donor nuclei are genetically altered to produce cloned embryos having desirable genetic traits.

18. (Amended) A method as claimed in claim 16, when used to produce an animal species of cloned embryo selected from the group comprising birds, amphibia, fish and mammals.

19. A method as claimed in claim 18, wherein said cloned animal embryo is a mammal, selected from the group comprising primates including humans, rodents, rabbits, cats, dogs, horses, cattle, sheep, deer, goats and pigs.

20. (Amended) A reconstituted non-human animal embryo prepared by the method claimed in claim 16.

21. (Amended) A reconstituted non-human animal embryo as claimed in claim 20, comprising a transgenic embryo.

22. (Amended) A reconstituted non-human animal embryo as claimed in claim 20 re-cloned to further increase embryo numbers or which undergoes serial nuclear transfer to aid nuclear reprogramming and/or development.

23. (Amended) A reconstituted non-human animal embryo as claimed in claim 20, comprising a species of mammal selected from the group comprising primates including humans, rodents, rabbits, cats, dogs, horses, cattle, sheep, deer, goats and pigs.

24. (Amended) A method of cloning a non-human animal comprising the steps: (1) producing a cloned non-human animal embryo according to the method of claim 16; (2) allowing a non-human animal to develop to term from the embryo; and (3) optionally breeding from the non-human animal so formed either by conventional methods or by further cloning.

25. A method as claimed in claim 24, wherein said cloned non-human animal is a non-human mammal selected from the group comprising non-human primates, rodents, rabbits, cats, dogs, horses, cattle, sheep, and deer.

26. (Amended) A method as claimed in claim 24, wherein said cloned non-human animal is a transgenic non-human animal having a desirable genetic trait.

27. (Amended) A method as claimed in claim 26, wherein said transgenic non-human animal is a transgenic bovine or ovine.

28. A cloned non-human animal prepared by the method of claim 24.

29. A cloned non-human animal as claimed in claim 28 comprising a mammal selected from the group comprising non-human primates, rodents, rabbits, cats, dogs, horses, cattle, sheep, and deer.

30. (Amended) A cloned non-human animal as claimed in claim 28, comprising a transgenic non-human animal having a desirable genetic trait.

31. A cloned non-human animal as claimed in claim 30 comprising a transgenic bovine or ovine.

32. (Amended) A cloned non-human transgenic animal as claimed in claim 30, wherein the desirable genetic trait is selected from the insertion, deletion, or modification of a gene or genes enabling the production of pharmaceutical proteins in milk, blood or urine; production of nutraceutical products in milk or meat; production of beneficial agricultural traits to improve the quality of milk, meat and fibre production; improve resistance to pests and diseases; production of industrial proteins in milk; xenotransplantation; and for the generation of transgenic animals as models for human disease.

33. (Amended) Offspring and descendants of the cloned non-human animal as claimed in claim 28.

34. (Amended) A method of producing an embryonic cell line comprising the steps a) selecting and segregating G1 cells from a proliferating population of donor cells or from a synchronised population of G1 cells or from a population of senescent cells, and transforming a nucleus from such a segregated cell into an enucleated recipient cell; b) growing to blastocyst stage; c) recovering embryonic cells; and d) establishing an immortalised cell line *in vitro*.

35. A method as claimed in claim 34, wherein said embryonic cells are embryonic stem cells.

36. (Amended) A method as claimed in claim 34, wherein said donor cells are human cells.

37. (Amended) A method as claimed in claim 34, wherein both donor and recipient cells are human cells.

38. (Amended) A method as claimed in claim 34, wherein the donor cells are adult or fetal cells selected from any karyotypically normal cell type and the recipient cells are selected from any cell type capable of reprogramming gene expression.

39. (Amended) An embryonic cell line produced by the method of claim 34.

40. (Amended) A human embryonic stem cell line produced by the method of claim 35, useful in therapeutic applications.

41. A method of producing embryonic stem cells comprising the steps of a) selecting and segregating G1 cells from a proliferating population of donor cells or from synchronised population of G1 cells or from a population of senescent cells and transferring a nucleus from

such a segregated cell into an enucleated recipient cell; b) growing to blastocyst stage; and c) recovering embryonic stem cells.

42. A method as claimed in claim 41, wherein said donor cells are human cells.

43. (Amended) A method as claimed in claim 41, wherein both donor and recipient cells are human cells.

44. (Amended) A method as claimed in claim 41, wherein the donor cells are adult or fetal cells selected from any karyotypically normal cell type and the recipient cells are selected from any cell type capable of reprogramming gene expression.

45. (Amended) Embryonic stem cells produced by the method of claim 41.

46. Embryonic stem cells as claimed in claim 45, comprising human embryonic stem cells.

49. (Amended) A method of therapeutic cloning, wherein embryonic stem cells are produced according to claim 35 from a donor cell derived from a subject, and cultured to produce specialised cells or tissue for transplantation in said subject or in another subject in need of such treatment.

50. A method as claimed in claim 49, wherein said embryonic stem cells comprise one or more transgenes to confer a desirable genetic trait in the resulting differentiated cells used for transplantation.

51. (Amended) A method of treating a disease, disorder or injury which may be treated by transplantation of specialised cells or tissue, comprising administering to a patient in need thereof a therapeutically effective amount of specialised cells or tissue produced according to the method of claim 49.

52. (Amended) A method as claimed in claim 49, wherein said disease, disorder or injury is selected from various neurological disorders (*eg* Parkinson's disease), diabetes, heart disease, muscular dystrophy, various hereditary diseases, specific cancers (*eg* leukemia), spinal cord injury, burns and other afflictions.

53. (Amended) A method of drug discovery or toxicology testing of drugs using *in vitro* differentiated human embryonic stem cells produced by the methods of claim 41.

54. (Amended) A method of xenotransplantation, wherein cells, tissues and organs are isolated from the non-human cloned animal of claim 28, and used for transplantation in a human patient in need thereof.

55. (Amended) A method of gene therapy, wherein cells, tissues and organs comprise a transgene and are isolated for the non-human cloned animal of claim 30.

EXHIBIT B
NATIONAL STAGE CLAIMS

1. A method of nuclear transfer, comprising selecting and segregating G1 cells from a proliferating or non-proliferating population of donor cells and transferring a nucleus from such a segregated G1 cell into an enucleated recipient cell.

2. A method as claimed in claim 1, wherein the donor cell population is at one or more known or unknown stages of the cell cycle.

3. (Amended) A method as claimed in claim 1 [or 2], wherein said donor cell population is non-proliferating and has been synchronised at any point in the G1 stage of the cell cycle.

4. (Amended) A method as claimed in [any one of claims 1 to 3] claim 1, wherein said G1 cell is segregated at an early G1 phase.

5. (Amended) A method as claimed in [any one of claims 1 to 3] claim 1, wherein the donor cell population is non-proliferating and comprises senescent cells.

6. (Amended) A method as claimed in [any one of claims 1 to 5] claim 1, wherein said donor cell population is derived from either embryo, fetal, juvenile or adult cells isolated from an animal *in vivo* or from a cell culture *in vitro*.

7. A method as claimed in claim 6, wherein said donor cell population comprises any diploid karyotypically normal cell capable of being stimulated to enter the cell cycle and proliferate.

8. A method as claimed in claim 7, wherein said donor cell population is of an undifferentiated cellular state or are at any degree of differentiation or quiescence or senescence.

9. (Amended) A method as claimed in [any preceding] claim 1, wherein the donor cells are adult or fetal fibroblasts or follicular cells.

10. (Amended) A method as claimed in [any preceding] claim 1, wherein said donor cells comprise modified cells.

11. A method as claimed in claim 10 wherein said donor cells comprise transgenic cells.

12. (Amended) A method as claimed in [any preceding] claim 1, wherein the recipient cell comprises an enucleated oocyte.

13. A method as claimed in claim 12, wherein the enucleated oocyte is obtained from a species corresponding in origin to the donor nuclei.

14. (Amended) A method as claimed in [any one of claims 1 to 11] claim 1, wherein the recipient cell comprises an enucleated stem cell or a clump of enucleated stem cells fused together.

15. A method as claimed in claim 14, wherein the stem cells are embryonic stem cells isolated from a growing embryo or form an established cell line in culture.

16. (Amended) A method of producing cloned animal embryos which comprises transferring a segregated donor nucleus in the G1 stage of the cell cycle into an enucleated recipient cell.

17. (Amended) A method as claimed in claim 16, wherein the donor nuclei are genetically altered [using methods well known in the art] to produce cloned embryos having desirable genetic traits.

18. (Amended) A method as claimed in claim 16 [or 17], when used to produce an animal species of cloned embryo selected from the group comprising birds, amphibia, fish and mammals.

19. A method as claimed in claim 18, wherein said cloned animal embryo is a mammal, selected from the group comprising primates including humans, rodents, rabbits, cats, dogs, horses, cattle, sheep, deer, goats and pigs.

20. (Amended) A reconstituted non-human animal embryo prepared by the method claimed in claim 16.

21. (Amended) A reconstituted non-human animal embryo as claimed in claim [17] 20, comprising a transgenic embryo.

22. (Amended) A reconstituted non-human animal embryo as claimed in claim 20 [or 21] re-cloned to further increase embryo numbers or which undergoes serial nuclear transfer to aid nuclear reprogramming and/or development.

23. (Amended) A reconstituted non-human animal embryo as claimed in [any one of claims 20 to 22] claim 20, comprising a species of mammal selected from the group comprising primates including humans, rodents, rabbits, cats, dogs, horses, cattle, sheep, deer, goats and pigs.

24. (Amended) A method of cloning a non-human animal comprising the steps: (1) producing a cloned non-human animal embryo according to the method of [any one of claim 16 or 17] claim 16; (2) allowing a non-human animal to develop to term from the embryo [by known methods]; and (3) optionally breeding from the non-human animal so formed either by conventional methods or by further cloning.

25. A method as claimed in claim 24, wherein said cloned non-human animal is a non-human mammal selected from the group comprising non-human primates, rodents, rabbits, cats, dogs, horses, cattle, sheep, and deer.

26. (Amended) A method as claimed in claim 24 [or 25], wherein said cloned non-human animal is a transgenic non-human animal having a desirable genetic trait.

27. (Amended) A method as claimed in claim 26, wherein said transgenic non-human animal is a transgenic bovine or ovine.

28. A cloned non-human animal prepared by the method of claim 24.

29. A cloned non-human animal as claimed in claim 28 comprising a mammal selected from the group comprising non-human primates, rodents, rabbits, cats, dogs, horses, cattle, sheep, and deer.

30. (Amended) A cloned non-human animal as claimed in claim 28 [or 29], comprising a transgenic non-human animal having a desirable genetic trait.

31. A cloned non-human animal as claimed in claim 30 comprising a transgenic bovine or ovine.

32. (Amended) A cloned non-human transgenic animal as claimed in claim 30 [or 31], wherein the desirable genetic trait is selected from the insertion, deletion, or modification of a gene or genes enabling the production of pharmaceutical proteins in milk, blood or urine; production of nutraceutical products in milk or meat; production of beneficial agricultural traits to improve the quality of milk, meat and fibre production; improve resistance to pests and diseases; production of industrial proteins in milk; xenotransplantation; and for the generation of transgenic animals as models for human disease.

33. (Amended) Offspring and descendants of the cloned non-human animal as claimed in [any one of claims 28 to 32] claim 28.

34. (Amended) A method of producing an embryonic cell line comprising the steps a) selecting and segregating G1 cells from a proliferating population of donor cells or from a synchronised population of G1 cells or from a population of senescent cells, and transforming a nucleus from such a segregated cell into an enucleated recipient cell; b) growing to blastocyst stage; c) recovering embryonic cells; and d) establishing an immortalised cell line *in vitro* [by methods known in the art].

35. A method as claimed in claim 34, wherein said embryonic cells are embryonic stem cells.

36. (Amended) A method as claimed in claim 34 [or 35], wherein said donor cells are human cells.

37. (Amended) A method as claimed in [any one of claims 34 to 36] claim 34, wherein both donor and recipient cells are human cells.

38. (Amended) A method as claimed in [any one of claims 34 to 37] claim 34, wherein the donor cells are adult or fetal cells selected from any karyotypically normal cell type and the recipient cells are selected from any cell type capable of reprogramming gene expression.

39. (Amended) An embryonic cell line produced by the method of [any one of claims 34 to 36] claim 34.

40. (Amended) A human embryonic stem cell line produced by the method of [claim 36, when dependent upon] claim 35, useful in therapeutic applications.

41. A method of producing embryonic stem cells comprising the steps of a) selecting and segregating G1 cells from a proliferating population of donor cells or from synchronised population of G1 cells or from a population of senescent cells and transferring a nucleus from such a segregated cell into an enucleated recipient cell; b) growing to blastocyst stage; and c) recovering embryonic stem cells.

42. A method as claimed in claim 41, wherein said donor cells are human cells.

43. (Amended) A method as claimed in [any one of claims 41 to 42] claim 41, wherein both donor and recipient cells are human cells.

44. (Amended) A method as claimed in [any one of claims 41 to 43] claim 41, wherein the donor cells are adult or fetal cells selected from any karyotypically normal cell type and the recipient cells are selected from any cell type capable of reprogramming gene expression.

45. (Amended) Embryonic stem cells produced by the method of [any one of claims 41 to 43] claim 41.

46. Embryonic stem cells as claimed in claim 45, comprising human embryonic stem cells.

Please cancel claims 47 and 48

. A use of the embryonic cells of any one of claims 39, 40 and 45, wherein specialised types of cell or tissue selected from the group comprising nerve cells, muscle cells, heart cells, liver cells, lung cells, kidney cells or any other type of cell of interest are cultured using methods well known in the art.

. A use as claimed in claim 47, wherein said embryonic cells are human embryonic stem cells as claimed in claim 40 or 46.

49. (Amended) A method of therapeutic cloning, wherein embryonic stem cells are produced according to [any one of claims 35 and 41 to 43] claim 35 from a donor cell derived from a subject, and cultured to produce specialised cells or tissue for transplantation in said subject or in another subject in need of such treatment.

50. A method as claimed in claim 49, wherein said embryonic stem cells comprise one or more transgenes to confer a desirable genetic trait in the resulting differentiated cells used for transplantation.

51. (Amended) A method of treating a disease, disorder or injury which may be treated by transplantation of specialised cells or tissue, comprising administering to a patient in need thereof a therapeutically effective amount of specialised cells or tissue produced according to the method of claim 49 [or 50].

52. (Amended) A method as claimed in claim 49 [or 50], wherein said disease, disorder or injury is selected from various neurological disorders (*eg* Parkinson's disease), diabetes, heart disease, muscular dystrophy, various hereditary diseases, specific cancers (*eg* leukemia), spinal cord injury, burns and other afflictions.

53. (Amended) A method of drug discovery or toxicology testing of drugs using *in vitro* differentiated human embryonic stem cells produced by the methods of claim [47] 41.

54. (Amended) A method of xenotransplantation, wherein cells, tissues and organs are isolated from the non-human cloned animal of [any one of claims 28 to 32] claim 28, and used for transplantation in a human patient in need thereof.

55. (Amended) A method of gene therapy, wherein cells, tissues and organs comprise a transgene and are isolated for the non-human cloned animal of claim 30 [or 31].

EXHIBIT C

REPLACEMENT PARAGRAPHS

At page 1, after the title, the additions are as shown:

The present application is a nationalization of International Patent Application PCT/NZ00/00179, filed September 14, 2000, which claims priority to New Zealand priority Application 337792, filed September 14, 1999.

Field of the Invention

The present invention concerns a novel method of nuclear transfer, specifically, although by no means exclusively for use in cloning technologies for the production of mammalian embryos, fetuses and offspring, including genetically engineered or transgenic mammalian embryos, fetuses and offspring.

At page 1, after the title, the final text is as follows:

The present application is a nationalization of International Patent Application PCT/NZ00/00179, filed September 14, 2000, which claims priority to New Zealand priority Application 337792, filed September 14, 1999.

Field of the Invention

The present invention concerns a novel method of nuclear transfer, specifically, although by no means exclusively for use in cloning technologies for the production of mammalian embryos, fetuses and offspring, including genetically engineered or transgenic mammalian embryos, fetuses and offspring.

After page 46, please start another page (47), and insert the following heading and the following text of the Abstract, as taken from the cover page of the PCT application:

ABSTRACT

The present invention provides a method of nuclear transfer by selecting and segregating G1 cells from a donor cell population. This method is advantageous over the prior art as it provides certainty as to the stage of the cell cycle which the donor nuclei are in and allows for the production of cloned transgenic or non-transgenic embryonic cells, reconstituted embryos and whole animals for agricultural, pharmaceutical, nutraceutical and biomedical applications.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: David WELLS.	§	
	§	Group Art Unit: Unknown
Serial No.: Unknown PCT/NZ00/00179	§	
	§	Examiner: Unknown
Filed: March 14, 2002	§	
Intl. Filing date: September 14, 2000	§	Atty. Dkt.: 4070.000300
Priority date: September 14, 1999	§	
For: NUCLEAR TRANSFER WITH SELECTED DONOR CELLS	§	

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March 14, 2002
Date

Rhonda Fairchild
Rhonda Fairchild

SUBMISSION OF FORMAL DRAWINGS

ATTN OFFICIAL DRAFTSMAN
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Applicant hereby submits the formal drawings for the above-referenced application (Figures 1-6 on 3 sheets) and request that these drawings be accepted for filing.

Respectfully submitted,

Shelley P.M. Fussey
Reg. No. 39,458
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Date: March 14, 2002

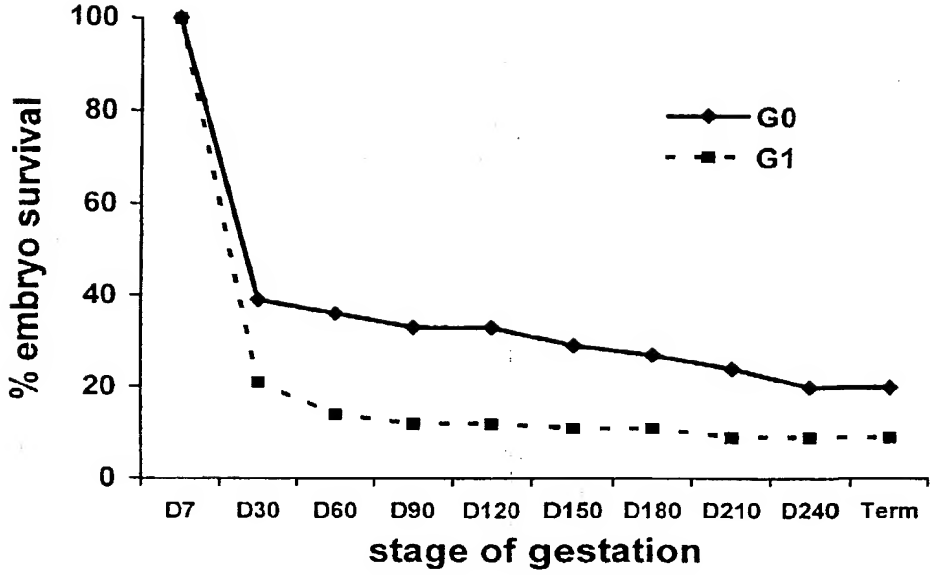
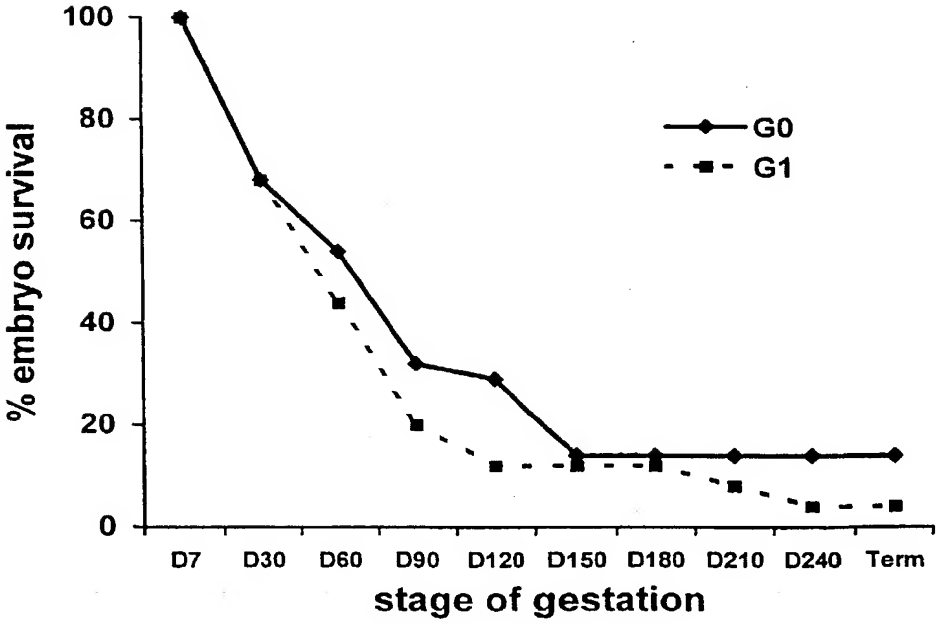


FIGURE 1



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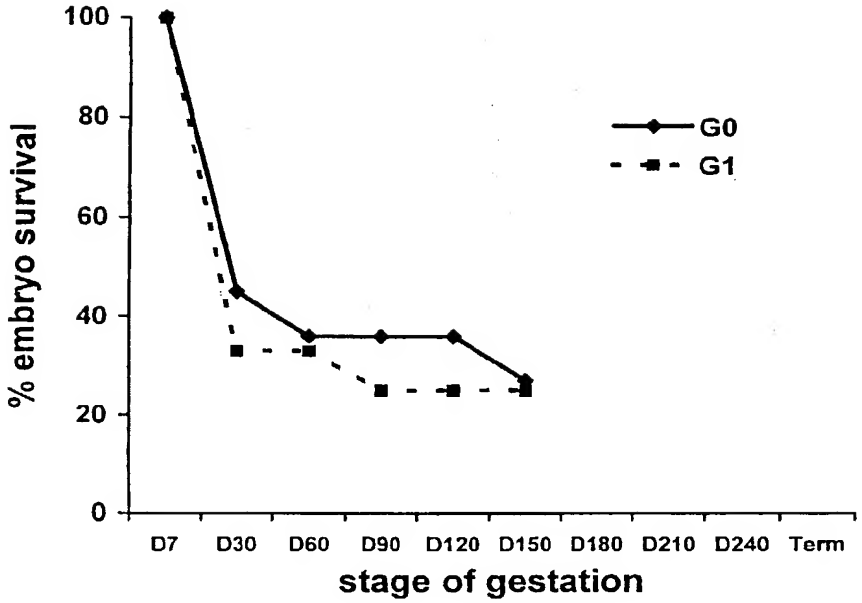

FIGURE 3

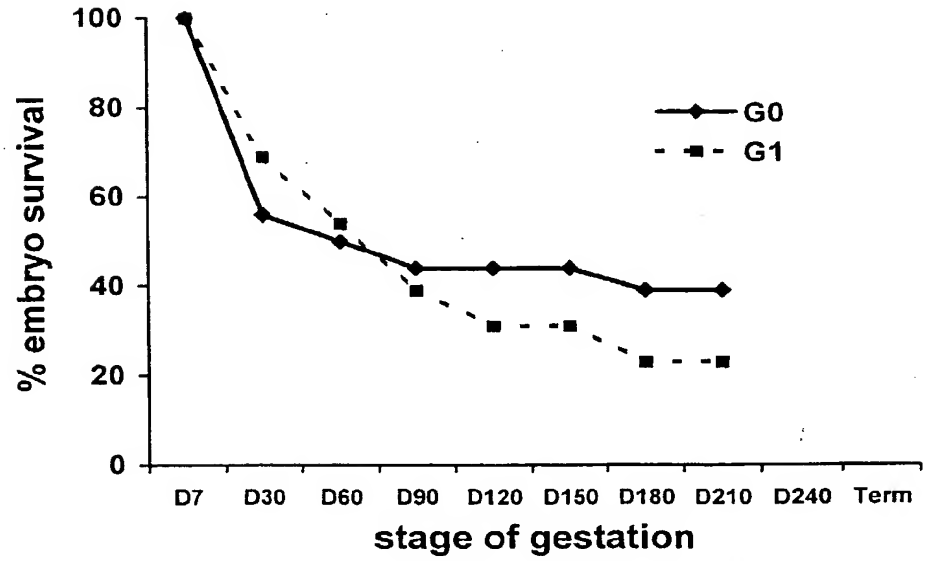

FIGURE 4

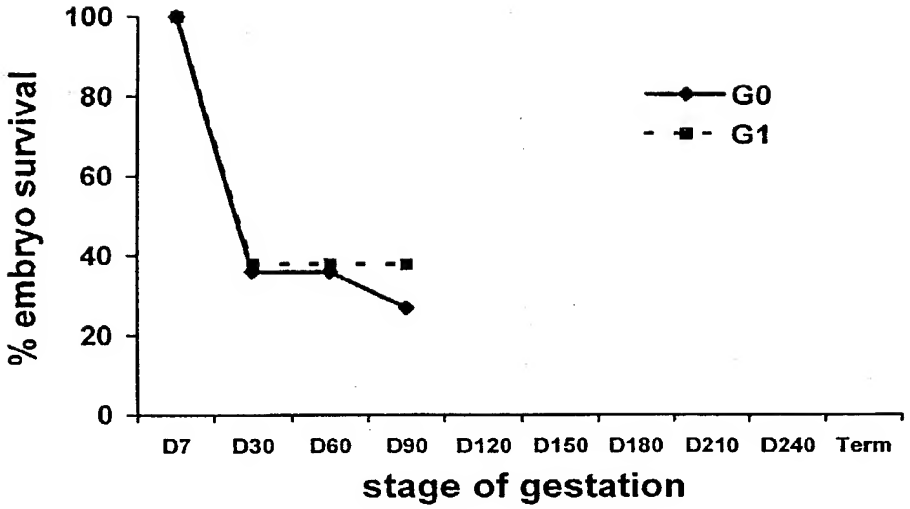

FIGURE 5

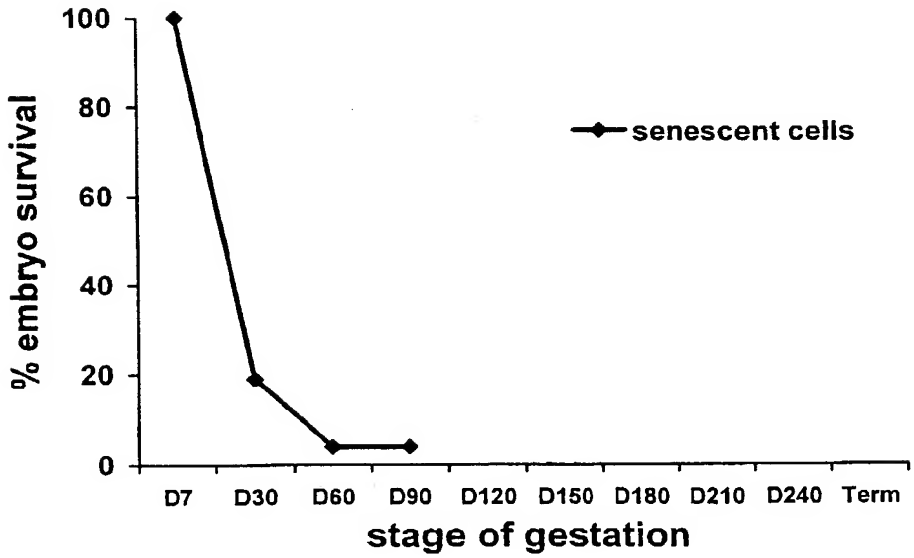

FIGURE 6

3/prts

NUCLEAR TRANSFER WITH SELECTED DONOR CELLS

The present invention concerns a novel method of nuclear transfer, specifically, although by no means exclusively for use in cloning technologies for the production of mammalian embryos, fetuses and offspring, including genetically engineered or transgenic mammalian embryos, fetuses and offspring.

BACKGROUND

The stage of cell cycle of the donor nucleus and the recipient cytoplast at the time of embryo reconstruction are important factors determining successful development following nuclear transfer. Specific combinations between the two "cells" are required to ensure a diploid set of chromatin following the first embryonic cell cycle and to maximise the opportunities for nuclear reprogramming and subsequent development. When an interphase donor nucleus is fused with an enucleated metaphase-arrested oocyte (termed the cytoplast or recipient cell), there is immediate nuclear envelope breakdown (NEBD) and the donor chromatin undergoes premature chromosome condensation (PCC)(Barnes *et al.*, 1993). These effects are induced by a cytoplasmic activity termed maturation promoting factor (MPF, alternatively called meiosis- or mitosis-promoting factor; see review by Campbell *et al.*, 1996a). MPF activity during oocyte maturation is maximal at metaphase stages and declines rapidly upon either fertilisation or artificial activation. Thus, two types of cytoplast may be used for reconstruction; those either high or low in MPF using either non-activated or activated metaphase II (MII) cytoplasts, respectively.

Early studies which helped gain an understanding of the importance of cell cycle co-ordination in mammalian nuclear transfer for maintaining chromosome integrity and hence developmental potential, were performed with undifferentiated blastomeres (*ie* non-specialised embryonic cells) from pre-implantation stage embryos in species such as rabbit, sheep and cattle. These studies revealed that the stage of the cell cycle of the donor nucleus and the length of exposure to MPF in the cytoplast have marked effects

on the degree of PCC observed. The chromatin of S-phase nuclei exposed to MPF has a typically pulverised appearance and chromosomal studies reveal a high incidence of abnormalities (Collas *et al.*, 1992b). In contrast, with nuclei at G1 or G2 the chromatin condenses to form elongated chromosomes with either single- or double-stranded chromatids, respectively (Collas *et al.*, 1992b). Following a suitable stimulus to release the reconstructed embryo from metaphase-arrest and to activate development, the nuclear envelope reforms around the donor chromatin which then undergoes DNA synthesis regardless of its previous cell cycle stage. Thus, donor nuclei- in G1 initiate DNA synthesis which is compatible with normal development, while nuclei in G2 or S-phases either completely or partially re-replicate already replicated DNA so that by the end of the first embryonic cell cycle the DNA content in the two daughter cells will be incorrect leading to abnormal early embryonic development.

In contrast, these early studies demonstrated that when blastomere nuclei are transferred to cytoplasts after the disappearance of MPF, following a sufficient interval after cytoplast activation, NEBD does not occur (so therefore neither does PCC) and it is the donor nucleus which controls DNA replication in accordance with its stage in the cell cycle at the time of transfer. Thus, nuclei in G1 or S-phases initiate or continue replication, respectively, while those in G2 are not induced to enter another round of DNA synthesis. Such pre-activated cytoplasts have been termed "universal recipients" (Campbell *et al.*, 1994) and are capable of co-ordinating the development of donor cells at any stage of the cell cycle. This has been especially important for cloning pre-implantation embryos where most undifferentiated blastomere nuclei are in S-phase at any one time (80-90%; Barnes *et al.*, 1993; Campbell *et al.*, 1994) and are therefore most compatible with transfer to cytoplasts low in MPF.

Following nuclear transfer, normal development will depend upon factors present within the oocyte cytoplasm (or additional factors introduced exogenously) being able to remodel chromatin structure and to appropriately reprogramme the pattern of gene expression of the donor nucleus. The mature cytoplast contains the RNA transcripts and proteins to direct development of the normally fertilised cleavage-stage embryo up

to the normal time of genome activation, when embryonic nuclei begin the synthesis of their own RNA to direct embryogenesis. Donor nuclei obtained from embryos or cell types which have already passed this point must therefore cease their RNA synthesis after reconstruction and remain inactive until the newly reprogrammed maternal-embryonic genome transition occurs. Following transfer, the donor nucleus is forced to reprogram to the zygotic state, and subsequently activate the appropriate genes at the correct levels, in the proper temporal and spatial manner for normal embryo development to occur. The mechanisms that achieve such nuclear reprogramming are currently not well understood.

Reprogramming and cell cycle co-ordination have become important topics given the recent interest in performing nuclear transfer with cells that can be maintained in culture. These cultured cells are more differentiated (*ie* possess a more specialised cellular function) that those used in the earlier studies with embryonic blastomere cloning. These cells can be isolated from either embryos, fetuses or adult animals. Because there is access to larger numbers of cells, efficient nuclear transfer techniques with these differentiated cell cultures would enable in livestock species large scale multiplication of desirable genotypes and would facilitate the production of transgenic animals following genetic manipulation of the cultured cells

Early studies with actively growing, unsynchronised cultures of ovine embryonic cells (cell cycle stage unknown) fused to pre-activated cytoplasts did in fact produce lambs at term from early (Campbell *et al.*, 1995) but not later passage cells (Campbell *et al.*, 1996b). Subsequent studies, where the cells were deprived of serum for 5 days and reported to be quiescent (*ie* whereby the cells supposedly exited the normal cell division cycle and entered a so-called "G0" state), yielded viable lambs following fusion with cytoplasts either before, after or simultaneous with activation at similar overall efficiencies (Campbell *et al.*, 1996b). These authors (Campbell *et al.*, 1996b; Schnieke *et al.*, 1997; Wilmut *et al.*, 1997; Patent WO 97/07669) suggest the importance of using cells that have exited the normal cell division cycle and are synchronised in a quiescent or G0 state to facilitate nuclear reprogramming and enable

the production of cloned animals from differentiated cells. The preferred method of synchronising cells in quiescence (based on absence of proliferating cell nuclear antigen (PCNA) indicating no cells in S-phase) by the above authors has been by serum starvation for a suitable period of time. Recently, however, it has been questioned as to what proportion of serum starved cells are actually in G0. Using dual parameter flow cytometry to simultaneously measure both cellular DNA and protein content (to distinguish between G0 and G1 cells in the diploid population, with quiescent cells having less RNA and protein) Boquest *et al.* (1999) investigated the cell cycle characteristics of cultured fetal pig fibroblasts. They demonstrated that despite serum starvation for 5 days, less than 50% of cells were actually in G0 by their definition. By selecting "small" cells in the population, the proportion in G0 increased to 72% in the serum starved cultures (Boquest *et al.*, 1999).

As an alternative to the use of quiescent cells, Cibelli and colleagues (1998; and patent specification WO 99/01163) reported the use of non-serum starved, randomly growing cultures of bovine cells fused to MII cytoplasts which were subsequently activated using ionomycin and 6-dimethylaminopurine (6-DMAP). However, it is not clear from these disclosures what the donor cell cycle stage was at the time of nuclear transfer in those resulting embryos that, ultimately yielded the cloned calves by these methods.

Similarly, there are other reports whereby cloned calves (Vignon *et al.*, 1998, 1999; Zakhartchenko *et al.*, 1999) and mice (Wakayama and Yangimachi, 1999) have been produced from non-serum deprived, randomly growing cell cultures. However, again, the stage of the donor cell cycle at the time of nuclear transfer which resulted in the cloned offspring remains uncertain.

Thus, none of these aforementioned studies have demonstrated exactly what stage or stages of the cell cycle have resulted in that low proportion of reconstructed embryos which ultimately develop into viable offspring.

The above discussion highlights that to date there has been generally poor characterisation as to the stage of the cell cycle cultured donor cells are in at the time of nuclear transfer, which makes the state of the art uncertain and not easily replicated.

It would therefore be desirable to have a method of nuclear transfer which ensured that the stage of cell cycle of the donor nuclei were accurately known.

It is an object of the present invention to go some way towards achieving this desideratum or at least to provide the public with a useful choice.

SUMMARY OF THE INVENTION

According to a first aspect, the present invention provides a method of nuclear transfer, comprising selecting and segregating G1 cells from a proliferating or non-proliferating population of diploid donor cells and transferring a nucleus from such a segregated G1 cell into an enucleated recipient cell, wherein donor cells are selected and segregated by physical picking based on individual cell identification to produce a pure G1 cell population. The donor cell population may be at one or more known or unknown stages of the cell cycle, with the proviso that said diploid donor cells are not selected from blastomeres which have been synchronised by ≥5μM nocodazole or 5 μg/ml colcemid.

Such a method provides certainty as to the stage of the cell cycle of the donor nuclei at the time of embryo reconstruction and is therefore advantageous over the prior art.

The invention contemplates the use of cell cycle inhibitors, (other than ≥5μM nocodazole or ≥5μM colcemid) to block randomly growing cells at specific stages of the cell cycle to produce a non-proliferating synchronised cell population. Preferably cell growth is blocked at mitosis and, following release from the inhibitor, cells progressing into G1 phase are used for nuclear transfer.

Thus, in a second aspect, the invention provides a method of nuclear transfer which comprises transferring a diploid nucleus from a cell segregated from a non-proliferating cell population which has been synchronised in the G1 stage of the cell cycle, into an enucleated recipient cell, with the proviso that said diploid donor cells are not selected from blastomeres which have been synchronised at G1-phase by ≥5μM nocodazole or 5 μg/ml colcemid.

Preferably said G1 cells are individually segregated from a randomly proliferating population or from a non-proliferating synchronised cell population at an early G1 phase.

Alternatively, the non-proliferation cell population may comprise senescent cells.

The segregated G1 donor cell is isolated from an animal *in vivo* or, more preferably, from a cell culture *in vitro*. Suitable cells may be derived from either embryos, fetuses, juvenile animals, through to fully mature adults. Practically any diploid karyotypically normal cell that is capable of cell proliferation or a senescent cell could be used in the current invention. Cells could be of an undifferentiated cellular state or at any varying degree of cellular differentiation so long as they can be stimulated to enter the cell cycle and proliferate. Cells which are quiescent could be stimulated to enter the cell cycle with the appropriate culture conditions (such as by the addition of serum or specific growth factors) and used for nuclear transfer in an early G1 state following mitosis. Some cell types may well prove to be more efficient than others, however, both adult and fetal fibroblasts and adult follicular cells have been found to be satisfactory. By way of demonstration of the invention, results are presented below in the bovine species (in examples 1-7) using two follicular cell lines, four skin fibroblast cell lines and two genetically modified fetal fibroblast cell lines.

Preferably the recipient cell comprises an enucleated oocyte obtained from a species corresponding in origin to the donor nuclei. The enucleated oocyte may have either high or low MPF activity at the time of embryo reconstruction as either state is compatible with a G1 donor nucleus with a 2C amount of DNA.

Alternatively, the recipient cell may comprise an enucleated stem cell or a clump of enucleated stem cells fused together. Preferably these stem cells are embryonic stem cells. Such embryonic stem cells that are used as the recipient cells in nuclear transfer may be themselves isolated from a growing embryo or from already established stem cell lines in culture. In this case, nuclear transfer using donor nuclei from G1 cells

selected by the method of the invention may be carried out for the purposes of "therapeutic cloning".

According to a third aspect, the present invention provides a method of producing cloned animal embryos by transferring a donor diploid nucleus from a cell selected and segregated in G1 phase according to the invention, preferably early G1 phase, into an enucleated recipient cell, with the proviso that said diploid donor cells are not selected from blastomeres which have been synchronised at G1-phase by ≥5μM nocodazole or 5 μg/ml colcemid.

The methods of the present invention may be used to produce any animal embryo species of interest including birds, amphibia, fish and mammals. Preferably the animal embryo of interest is a mammal, including, but not limited to, primates including humans, rodents, rabbits, cats, dogs, horses, pigs and most preferably, ungulates such as cattle, sheep, deer and goats.

Preferably the cloned animal embryos have desirable genetic traits using genetically modified nuclei by methods known in the art.

According to a fourth aspect, the present invention provides a reconstituted animal embryo prepared by the methods of the invention including a reconstituted transgenic animal embryo. The embryos so formed may then be either re-cloned to further increase embryo numbers or undergo serial nuclear transfer to further aid nuclear reprogramming and/or developmental potential.

According to a fifth aspect, the present invention provides a method of cloning a non-human animal comprising (1) producing a cloned animal embryo according to the method of the invention described above; (2) allowing an animal to develop to term from the embryo by known methods; and (3) optionally breeding from the animal so formed either by conventional methods or by cloning according to the methods of the present invention.

The methods of the present invention may be used to produce a non-human animal species of interest including birds, amphibia, fish and mammals. Preferably said non-

human animal is selected from the group comprising non-human primates, rodents, rabbits, cats, dogs, horses, pigs and most preferably ungulates such as cattle, sheep, deer and goats.

According to a sixth aspect, the present invention provides a cloned non-human animal prepared by the methods of the invention, described above, as well as to the offspring and descendants of such cloned non-human animals.

The methods of the present invention may be used to produce non-human animals, preferably mammals, having desirable genetic traits using genetically altered donor nuclei by methods well known in the art. Transgenic animals produced by such methods also form part of the present invention.

The present invention can also be used to produce embryonic cell lines, embryonic stem cells, fetuses or offspring which can be used, for example, in cell, tissue and organ transplantation.

Accordingly, in a further embodiment, the present invention provides a method of producing a cell line comprising the steps a) selecting and segregating G1 cells from a proliferating population of diploid donor cells of unknown cell cycle or from a synchronised population of diploid G1 cells and transferring a nucleus from such a segregated cell into an enucleated recipient cell; b) optionally growing to embryo stage; c) recovering cells; and d) establishing an immortalised cell line *in vitro* by methods known in the art.

In a further embodiment there is provided a method of producing animal stem cells comprising the steps of a) selecting and segregating G1 cells from a growing population of animal diploid donor cells at various unknown stages of the cell cycle, or from synchronised cultures of diploid G1 cells, and transferring the nucleus from such segregated cells into an enucleated animal recipient cell; b) optionally growing to embryo stage; c) recovering stem cells.

Preferably, the stem cells are embryonic stem cells.

Preferably, the animal donor cell used in the above methods is of human origin. Most preferably, both the animal donor and recipient cells used in the above methods are of human origin.

Most preferably, the donor cells are adult or fetal cells selected from any karyotypically normal cell type and the recipient cells are selected from any cell type capable of reprogramming gene expression including enucleated oocytes or embryonic stem cells.

The embryonic stem cells produced by the method of the invention are pluripotent and may be induced to differentiate in culture to form purified populations of specialised types of human cells including nerve cells such as neurons, astrocytes, oligodendrocytes; liver cells; muscle cells such as myocytes; heart cells such as cardiomyocytes, haematopoietic cells, pancreatic cells and any other cell type of interest by methods known in the art.

Such specialised human cells and tissues may then be used for transplantation for treatment of specific diseases or injuries where the damaged cells are unable to replace themselves or replace themselves effectively. Where the human donor cell was derived from a patient in need of such a transplant, such a transplanted tissue would not be rejected by the patient as the tissue would be genetically identical to the patient.

Alternatively, such differentiated cells and tissues could be used to treat diseases or injuries, for example, various neurological disorders (*eg* Parkinson's disease), diabetes, heart disease, muscular dystrophy, various hereditary diseases, specific cancers (*eg* leukemia), spinal cord injury, burns and other afflictions.

These methods are known as "therapeutic cloning" in the art.

The *in vitro* differentiation of human embryonic stem cells to specific cell types may be also beneficial for drug discovery and toxicology studies for human medicine.

Thus, in a further aspect, the present invention provides a method of drug discovery and toxicology testing of drugs using *in vitro* differentiated human embryonic stem cells produced by the methods of the present invention.

According to a still further aspect, the present invention provides a method of xenotransplantation whereby cells, tissues and organs may be isolated from cloned non-human animals and their offspring produced according to the methods of the invention, and used for transplantation in human patients in need of such therapy. Where such cells, tissues or organs comprise a transgene, such cells, tissues or organs may be useful in gene therapy or to moderate the patient's immune response to the xenogenic tissue.

DESCRIPTION OF THE FIGURES

The present invention will now be described by reference to the figures of the accompanying drawings in which:

Figure 1 shows the survival rate throughout the gestation of cloned cattle embryos reconstructed with follicular cells either in G0 or G1 of the donor cell cycle;

Figure 2 shows the survival rate throughout gestation of cloned cattle embryos reconstructed with adult female skin fibroblasts either in G0 or G1 of the donor cell cycle;

Figure 3 shows the survival rate throughout gestation of cloned cattle embryos reconstructed with adult female skin fibroblasts (3XTC cells) either in G0 or G1 of the donor cell cycle;

Figure 4 shows embryonic survival throughout gestation of cloned cattle embryos reconstructed with adult male skin fibroblast cells (LJ801 cells) either in G0 or G1 of the donor cell cycle;

Figure 5 shows embryonic survival throughout gestation of cloned transgenic cattle embryos reconstructed with genetically modified female fetal lung fibroblast cells (casein plus 5110 cells) either in G0 or G1 stages of the donor cell cycle; and

Figure 6 shows embryonic survival throughout gestation of cloned transgenic cattle embryos reconstructed with non-proliferating, senescent female fetal fibroblasts (561 cell line).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to an improvement of the known techniques for the cloning of mammalian embryos by nuclear transfer. Although it is contemplated that the embryo cloning procedure of the present invention may be utilised in a variety of mammals and, indeed other animal species, the procedure will be described with reference to the bovine species. It is an essential feature of this invention that the donor nuclei are diploid and are in G1 phase, more preferably in early G1 phase.

One approach in obtaining G1 cells with certainty from a randomly growing population of cultured cells is to individually pick mitotic cells from the culture surface, and allow them to complete mitosis in medium containing 10% fetal calf serum (FCS). Such donor cells are then fused to recipient cells, (*ie* cytoplasts) within a short period of time following mitosis, such as three hours in common practice and before entry into S-phase, as may be detected by BrdU labeling. In this way they are assured of being in the early phase of G1 and possess a 2C amount of DNA. Thus, the cycling cells are used for nuclear transfer before they have progressed to the G1/S boundary. The selected cells remain in high serum-containing medium throughout the manipulations,

at least until after fusion with the cytoplast has been completed. Thus, the cells are not induced to exit the cell division cycle and do not become quiescent at any point.

Although the present invention contemplates the production of a synchronised population of G1 cells for use in nuclear transfer, one advantage of the methods of the present invention is that they do not necessitate the use of potentially cyto-toxic or perturbing cell synchronisation agents such as nocodazole or colchicine to, for example, pre-synchronise a higher proportion of cells in M-phase before subsequent release from this block and selection of cells in early G1 following cell division. However, in order to select larger numbers of G1 cells or to reversibly arrest cells at specific points during the G1 phase for use in nuclear transfer, it may be advantageous, according to the present invention, to utilise suitable reagents i.e. cell cycle inhibitors at appropriate drug concentrations and incubation times. Such reagents and methods will be known to people skilled in the art. Methods may include a pre-synchronisation treatment to reduce drug exposure times, by for example, temporarily inducing cells to enter G0 by serum deprivation before re-adding serum and allowing the cells to re-enter at the G0/G1 boundary or restriction point and progress through the cell division cycle. Suitable reagents to reversibly arrest cells in various points in G1 (as shown in Gadbois *et al.*, 1992 and references therein) include: (1) staurosporine (a non-specific kinase inhibitor used at extremely low concentrations in the nano-molar range); (2) more specific kinase inhibitors on cell cycle progression, such as for example inhibitors of cAMP-dependent protein kinase and cGMP-dependent protein kinase; (3) lovastatin; (4) isoleucine deficiency; and (5) aphidicolin or hydroxyurea used in a manner to prevent entry into S-phase and block cells at the G1/S boundary.

Since G1 donor nuclei have a 2C amount of DNA (i.e. it is diploid) they may be reconstructed with cytoplasts possessing either a high or low amount of MPF. That is to say, G1 nuclei may be introduced into cytoplasts either before, after or at the same time as activation occurs. However, for improved development of the cloned embryos it is preferable to expose the donor nucleus (introduced either following electrically-induced cell fusion or direct nuclei injection) to factors present within the cytoplasm of

the enucleated oocyte for a suitable period of time in order to facilitate nuclear reprogramming. This has been termed "fusion before activation" or FBA. Previous work has demonstrated the benefits of this approach compared to essentially "simultaneous fusion and activation" or AFS (Stice *et al.*, 1996; Wells *et al.*, 1998; 1999). Furthermore, it is recommended that exposure to the cytoplasm be at least greater than one hour duration and preferably between 3-6 hours in order to improve rates of development to the blastocyst-stage. With this method, however, it is important to prevent by some suitable means the micro-nuclei formation which occurs when fusion precedes activation (Czolowska *et al.*, 1984) in order to maintain the correct ploidy in the resulting embryo.

Below is outlined a method for reconstructing and producing cloned embryos, in the bovine species, derived from both G0 (control) and G1 cultured donor cells. In this particular example described below, bovine follicular cells collected from ovarian follicles were used with results presented in examples 1-3 (the use of fibroblast cell lines is illustrated in examples 4-7). In practice, essentially any cell type possessing a normal diploid karyotype, including embryonic, fetal, juvenile and adult cells, which is either proliferating or can be induced to enter the cell cycle or senescent may prove totipotent using this technology. In addition, any other method known in the art may be used to reconstitute and produce cloned embryos as would be appreciated by a person skilled in the art.

In Vitro Maturation of Oocytes

Slaughterhouse ovaries were collected from female cattle and placed in saline (30° C) and transported within 2 hours to the laboratory. Cumulus-oocyte complexes (COCs) were recovered by aspiration of 3-10 mm follicles using an 18-gauge needle and negative pressure. (Alternatively, immature oocytes could be collected from donor cows via ovum pick-up and subsequently matured *in vitro*). COCs were collected into HEPES-buffered Tissue Culture Medium 199 (H199; Life Technologies, Auckland, New Zealand) supplemented with 50 µg/ml heparin (Sigma, St. Louis, MO) and 0.4%

w/v BSA (Immuno-Chemical Products (ICP), Auckland, New Zealand). Before *in vitro* maturation, only those COCs with a compact, non-atretic cumulus oophorus-corona radiata and a homogenous ooplasm were selected. They were washed twice in H199 medium + 10% FCS (Life Technologies) before being washed once in bicarbonate-buffered Tissue Culture Medium 199 medium + 10% FCS. Ten COCs were transferred in 10 µl of this medium and placed into a 40 µl drop of maturation medium in 5-cm petri dishes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) overlaid with paraffin oil (Squibb, Princeton, NJ). The maturation medium comprised Tissue Culture Medium 199 supplemented with 10% FCS, 10 µg/ml ovine FSH (Ovagen; ICP), 1 µg/ml ovine LH (ICP), 1 µg/ml oestradiol (Sigma), and 0.1 mM cystamine (Sigma). Microdrop dishes were cultured at 39° C in a humidified 5% CO_2 in air atmosphere for 18-20 hours. After maturation, the cumulus-corona was totally removed by vortexing COCs in 0.1% hyaluronidase (from bovine testis; Sigma) in HEPES-buffered Synthetic Oviduct Fluid (HSOF; Thompson *et al.*, 1990) for 3 minutes, followed by three washes in HSOF + 10% FCS.

Nuclear Transfer with Cultured Cells

a) Media Matured oocytes, cytoplasts and reconstructed embryos were either held or manipulated in H199-based media for the period following maturation and until fusion was assessed 15-30 minutes after the electrical pulses. Embryos that were reconstructed by fusing donor cells and MII cytoplasts 3-6 hours before activation (FBA treatment) were cultured in AgResearch Synthetic Oviduct Fluid medium (AgR SOF; which is a modified formulation to that described in Gardner *et al.*, 1994, and is commercially available from AgResearch, Hamilton, New Zealand) minus calcium + 10% FCS until just before activation some 3-6 hours later. Following this point, calcium was present in all media formulations used.

b) Enucleation Oocytes matured for approximately 18-20 hours were enucleated with a 15-20 µm (external diameter) glass pipette, by aspirating the first polar body and the MII plate in a small volume of surrounding cytoplasm. The oocytes were

previously stained in H199 medium containing 10% FCS, 5 μg/ml Hoechst 33342 and 7.5 μg/ml cytochalasin B (Sigma) for 5-10 minutes and manipulated in this medium but without Hoechst 33342. Enucleation was confirmed by visualising the karyoplast under ultraviolet light. Following enucleation, the resulting cytoplasts were washed extensively in H199 + 10% FCS and held in this medium until injection of donor cells.

c) Preparation of quiescent (G0) donor cells Cultured follicular cells were induced to enter a period of quiescence by serum deprivation (Campbell *et al.*, 1996b). One day after routine passage, the culture medium was aspirated and the cells washed three times with fresh changes of PBS before fresh culture medium containing only 0.5% FCS was added. The follicular cells were returned to culture for a further 9-23 days (commonly 10 days) in low serum before they were used for nuclear transfer. Immediately before injection, a single cell suspension of the donor cells was prepared by standard trypsinization. The cells were pelleted and resuspended in H199 + 0.5% FCS and remained in this medium until injection.

d) Preparation of G1 donor cells Proliferating follicular cells were cultured in an appropriate medium (for example DMEM/F12 plus 10% fetal calf serum) on glass coverslips in the culture dish. These coverslips containing the cells growing on their surface may be physically picked out of the culture dish in a sterile manner and placed into a suitably constructed micro-manipulation chamber, to enable cell or nuclei collection and injection. A droplet of HEPES-buffered medium containing 10% FCS is placed onto the cells which is then overlaid with mineral oil. So long as the density of the cells on the coverslip is not too high, it is possible to identify and with care, physically pick the mitotic cells off the coverslip with a manipulation pipette since they have rounded up and are only loosely attached to the culture surface during mitosis. If this proves difficult, one may briefly wash the cells with PBS before introducing a dilute concentration of trypsin solution (such as at one-tenth the strength used for routine sub-cultivation of the cultured cell line) containing 1.5 μg/ml cytochalasin B, principally to minimise any mechanical damage induced during physical removal of the cells from the coverslip. With suitable microscopy (for example phase contrast or

DIC optics) mitotic cells are identified on the coverslip primarily by visualising condensed chromatin on a mitotic spindle or by identifying condensed chromatin in a cell doublet, still connected by a cytoplasmic bridge, undergoing telophase stage of mitosis. Thus, there is no need to use a DNA specific flurochrome such as Hoechst 33342 and expose cells to UV light. With the aid of the injection pipette mounted on the manipulator, these mitotic cells are individually picked off the coverslip and placed into an adjacent droplet of HEPES-buffered medium containing 10% FCS to enable complete mitosis and eventual cell cleavage to form a doublet of cells. The diameter of the pipette should be of a suitable size, dependent upon the cell line, so as to not physically damage the cell or the spindle during manipulation. Thus, mitotic cells preferably in anaphase or telophase stages are individually selected, removed and allowed to complete mitosis and cleave in two. These cell doublets are then gently separated into individual cells which may be easily achieved by brief exposure to a suitable enzymatic solution. Each intact cell is then injected and fused to the cytoplast. Alternatively the cell nucleus may be isolated and injected directly into the cytoplasm of the enucleated oocyte. Preferably the introduction of the donor nucleus into the cytoplast is completed within three hours of originally picking the mitotic cell off the culture surface. This ensures that the cultured cells are fused at an early G1 stage of the cell cycle and before S-phase occurs. With each individual cell type or cell line used this should be confirmed by, for example, negative BrdU labeling of a sample of the selected cells. In addition, confirmation that the cells so picked by this method are in fact cycling and do enter S-phase at some latter point is recommended.

e) **Microinjection** Recipient cytoplasts were dehydrated in H199 containing 10% FCS and 5% sucrose. This medium was also used as the micro-manipulation medium. A suitably-sized pipette (e.g. 30-35 μm external diameter), containing the donor cell, was introduced through the zona pellucida and the cell wedged between the zona and the cytoplast membrane to facilitate close membrane contact for subsequent fusion. Following injection, the reconstructed embryos were rehydrated in two steps; firstly in H199 containing 10% FCS and 2.5% sucrose for 5 minutes and then in H199 + 10% FCS until fusion.

f) Cell fusion For both the G0 and G1 cell treatments embryos were reconstructed using the FBA (fusion before activation) strategy. Reconstructed embryos were electrically fused at approximately 24 hours post-start of maturation (hpm) in buffer comprising 0.3 M mannitol, 0.5 mM HEPES and 0.05% fatty acid free (FAF) BSA with 0.05 mM calcium and 0.1 mM magnesium. Fusion was performed at room temperature, in a chamber with two stainless steel electrodes 500 µm apart overlaid with fusion buffer. The reconstructed embryos were manually aligned with a fine, mouth-controlled Pasteur pipette, so that the contact surface between the cytoplast and the donor cell was parallel to the electrodes. For the follicular cells, cell fusion was induced with two DC pulses of 2.25 to 2.50 kV/cm for 15 µs each, delivered by a BTX Electrocell Manipulator 200 (BTX, San Diego, CA) (optimal electrical parameters need to be identified for each cell line). Following the electrical stimulus, the reconstructed embryos were washed in H199 + 10% FCS. They were then checked for fusion by microscopic examination within 15-30 minutes.

The electrical fusion parameters above are not expected to cause significant rates of activation with young cytoplasts used at 24 hpm, since less than 1% of control oocytes (n=112) at the same age formed pronuclei after a similar electrical stimulus (Wells *et al.*, 1999). This is important for the FBA treatment, so that NEBD and PCC do occur, allowing exposure of the donor chromatin of the G1 and G0 nuclei to factors present within the oocyte cytoplasm to enable chromatin remodeling and nuclear reprogramming.

Alternatively, the nuclei from cells in G1 may be isolated and injected directly into the oocyte cytoplasm as known by a person skilled in the art.

g) Activation There are a variety of methods to effect artificial activation. One particular method involves the combination of ionomycin (Sigma) and 6-dimethylaminopurine (6-DMAP; Sigma) (Susko-Parrish *et al.*, 1994). Following fusion, embryos were activated preferably after the donor nuclei have been exposed to

the oocyte cytoplasm for a period of 3-6 hours. This preferred method has been termed "fusion before activation" (FBA; Wells *et al.*, 1998). Thirty minutes before activation, fused embryos in the FBA treatment were washed and held in HSOF (containing calcium) + 1 mg/ml FAF BSA. Activation was induced by incubation in 30-µl drops of 5 µM ionomycin (Sigma) in HSOF + 1mg/ml FAF BSA for 4 minutes at 37° C. Activation commonly occurred in cytoplasts aged between 27-30 hpm. Embryos were then extensively washed in HSOF + 30 mg/ml FAF BSA for 5 minutes before culture in 2 mM 6-dimethylaminopurine (6-DMAP; Sigma) for 4 hours in AgR SOF (plus calcium) + 10% FCS.

The improved rates of embryo development that result from a period of prolonged exposure of the nucleus to oocyte cytoplasm as in the FBA methodology (Stice *et al.*, 1996; Wells *et al.*, 1998; 1999) must be combined with suitable treatments to prevent micronuclei formation occurring following such delayed activation (Czolowska *et al.*, 1984). A serine-threonine kinase inhibitor such as 6-DMAP appears to be one suitable reagent. 6-DMAP therefore allows for the formation of a single intact nucleus following the initial activation stimulus thus, maintaining the correct ploidy in the reconstructed embryo.

In Vitro Culture of Nuclear Transfer Embryos

Embryo culture was performed in 20 µl drops of AgR SOF (commercially available from AgResearch, Hamilton, New Zealand) overlaid with paraffin oil. AgR SOF is a modified formulation of SOFaaBSA (containing 8 mg/ml FAF BSA; as described by Gardner *et al.*, 1994). Whenever possible, groups of up to 10 embryos were cultured together in droplets of medium. Embryos were cultured in a humidified modular incubator chamber (ICN Biomedicals, Aurora, OH) at 39° C in a 5% CO_2: 7% O_2: 88% N_2 gas mix. On Day 4-5 of development (Day 0 = day of embryo reconstruction), embryos were transferred to fresh 20 µl drops of AgR SOF plus 10 µM, 2, 4-dinitrophenol acting as an uncoupler of oxidative phosphorylation to improve the *in vitro* development of bovine embryos as taught in published patent specification No.

WO 00/38583, which is incorporated herein by reference. On Day 7 post fusion, development to transferable-quality blastocysts was assessed.

Embryo Transfer, Pregnancy Diagnoses and Calving

The transfer of embryos, diagnosis of pregnancy and calving management were carried out using skills well known in the art.

Serial Nuclear Transfer and Recloning

In a further embodiment of the invention, it may be desirable to reclone the first generation cloned embryos originally produced by reconstructing a donor cell in G1 with a suitable recipient cell. Recloning may be achieved by disaggregating a pre-implantation stage embryo into individual cells and then fusing each of these to suitable recipient cytoplasts. As would be appreciated by a person skilled in the art, this form of embryonic cell (or blastomere) cloning requires co-ordination of the cell cycles between the donor and recipient cells to avoid chromosomal abnormalities and optimise development. Preferably donor cells are obtained when the first generation cloned embryo is at the morula stage (approximately 32 cells in cattle) but the embryo could be at either earlier or later stages of development. Alternatively, one could firstly generate a cloned fetus produced from a G1 cultured donor cell, then re-derive a fetal cell line and reclone embryos using this new cell line. This recloning approach may provide an advantage in nuclear reprogramming by prolonging the period of exposure of the original donor nucleus to oocyte cytoplasm during the early pre-implantation period. It also has the advantage of multiplying the numbers of cloned embryos available from the first generation founder embryo, to produce cloned embryos in the second generation, third generation and so on.

Serial nuclear transfer involves the sequential transfer of nuclei to suitable recipient cell cytoplasmic environments. As an embodiment of the present invention, it may be desirable to firstly reconstruct a one-cell embryo with a G1 donor cell and a non-

activated recipient cytoplast high in MPF. This will allow NEBD and PCC and when followed by an activation stimulus, an intact diploid nucleus will be formed. This nucleus (that has undergone nuclear remodeling and a degree of nuclear reprogramming) may then be aspirated as a karyoplast from the one-cell cloned embryo and be sequentially transferred to an enucleated zygote at an appropriate stage post-fertilisation. Embryo development is then allowed to proceed. Such a sequential serial nuclear transfer process may improve nuclear reprogramming. It may also result in improved development as a result of the second nuclear transfer step introducing the nucleus into a cytoplasmic environment which has been more appropriately activated into embryonic development as a consequence of having undergone fertilisation with a sperm.

Production of Transgenic Animals

The production of transgenic livestock following nuclear transfer with genetically modified donor nuclei at the G1 stage of the cell cycle will likely be a more efficient and versatile approach that pronuclear injection of DNA into zygotes (Wall *et al.*, 1997) or sperm-mediated transgenesis involving intra-cytoplasmic injection of sperm and exogenously-bound DNA (Perry *et al.*, 1999). The advantages of the nuclear transfer approach with cultured cells include; (1) a far wider range of genetic manipulations are possible; (2) it more readily enables genetic manipulation on a high genetic background using a cell line as opposed to collecting oocytes or zygotes; (3) all of the resulting cloned offspring are transgenic and of the desired sex; and (4) there is the opportunity to produce instant flocks and herds generating useful product in a shorter timeframe compared to producing individual founder animals with the pronuclear injection approach or sperm-mediated transgenesis.

Cultured cells may be genetically altered by any known method to insert, remove or modify a desired DNA sequence. Modifications include the random insertion of new DNA sequences (which may be heterologous), site specific insertion of DNA and homologous recombination which allow the insertion, deletion or modification of a

DNA sequence at a specific site in the genome. Following cell selection and DNA analyses to verify the desired genetic modification using methods known in the art, karyotypically normal transgenic cells may then be selected in G1 phase of the cell cycle and used for nuclear transfer to generate cloned / transgenic animals.

There are a wide variety of opportunities available for genetically modifying livestock for both biomedicine and agriculture, depending upon the specific genes that are manipulated. The areas of opportunity include; (1) production of pharmaceutical proteins in milk, blood or urine; (2) production of nutraceutical products and medical foods, for example in milk; (3) manipulation of agricultural production traits, for example improving the quantity and quality of milk, meat and fibre and improving disease and pest resistance, (4) production of industrial proteins in milk, for example; (5) xenotransplantation; (6) generating livestock as models for human disease, for example cystic fibrosis and Huntington's disease.

Therapeutic Cloning

A significant impact of nuclear transfer and embryonic stem cell technologies may be in the area of human cell-based therapies (Pedersen, 1999). Patients with particular diseases or injuries in tissues which neither repair nor replace themselves effectively (as occurs in diabetes, muscular dystrophy, spinal cord injury, certain cancers and various neurological disorders, including Parkinson's disease, etc) could potentially generate their own therapeutic tissue for transplantation, offering prolonged or lifelong treatment. Initially, this approach would employ human nuclear transfer. This may involve collecting a small sample of healthy tissue from a human patient suffering a particular disease or injury and stimulating the proliferation of cells in culture. By selecting donor cells in G1 of the cell cycle and fusing them to a suitable recipient cell it may be possible to reprogramme the nucleus. If the recipient cell were an enucleated human oocyte then the reconstructed embryo, following a suitable activation stimulus, could be grown in an appropriate embryo culture medium to the blastocyst stage. Under suitable culture conditions (Thomson *et al.*, 1998) human embryonic stem cells

may then be derived from the inner cell mass of such a cloned embryo which would be genetically identical to the patient which donated the cultured cells.

By "embryonic stem cells" we mean cells isolated from any pluripotent cell types present within the embryo, and are preferably isolated from the inner cell mass of the blastocyst stage embryo by methods well known in the art.

The embryonic stem cells so produced by the method of the invention would be undifferentiated, pluripotent (potentially capable of differentiating into almost any cell type in the body) and possess essentially unlimited proliferative capacity *in vitro*. Alternatively, cell lines isolated from the inner cell mass may be somewhat more differentiated and possess a more limited ability to differentiate into a wide variety of cell types, but may still be therapeutically useful. Based on experience with mouse embryonic stem cells, suitable conditions can be developed which enable the production of purified populations of specific differentiated cell types such as nerve cells, haematopoietic cells, cardiomyocytes, etc to treat specific disorders (*eg* insulin-producing pancreatic cells for diabetes or dopamine-producing nerve cells for Parkinson's disease). These genetically compatible cells could then be administered back to the human patient, in order to regenerate normal tissue *in situ* following transplantation. Because the cells are genetically identical to the patient they will not be rejected and so there would be no or little need for immuno-suppressive drugs. It may also prove beneficial to generate "universal" embryonic stem cell lines for allogenic transplantation following systematic modification of loci such as major histocompatability complex genes that play an important role in the recognition of foreign cells by the immune system.

Some applications may involve the genetic modification of the embryonic stem cells prior to differentiation and transplantation. This may be for the purposes of gene therapy to deliver a therapeutic drug for treatment or to correct a genetic defect in somatic cells such as occurs in the dystrophin gene in the skeletal muscle of patients with Duchenne muscular dystrophy.

The differentiation of human embryonic stem cells to specific cell types may also be beneficial for drug discovery and toxicology studies for human medicine, in addition to transplantation therapy with cells, tissue or organs.

In addition, cells, tissues and organs may be isolated from cloned non-human animal offspring, and used for transplantation in human patients in need of such therapy (xenotransplantation). Where such cells, tissues or organs comprise a transgene, such cells, tissues or organs may be useful in gene therapy or to moderate the patient's immune response to the xenogenic tissue.

Recipient cells for human applications

In the method of nuclear transfer according to the invention, the preferred recipient is an enucleated oocyte prepared by the method disclosed above. However, for human applications this may prove difficult.

An alternative source of recipient cells to reprogramme differentiated somatic nuclei may be embryonic stem cells. Thus, somatic cells from a patient requiring some form of cell-based therapy may be de-differentiated in culture without the requirement of human oocytes. This may be achieved by fusing a healthy somatic cell in G1 of the cell cycle to an enucleated embryonic stem cell or a group of embryonic stem cells (to provide a larger mass of cytoplasm for reprogramming). It would be necessary to control the state of the cell cycle of the recipient stem cells, preferably this would be at M-phase or G1-phase at the time of fusion. In this way, the differentiated nucleus of the somatic cell may be de-differentiated following exposure to the cytoplasm of the stem cell. The resulting reconstructed cell may have multipotent or pluripotent developmental potential, and may be induced to form an array of other specialised cell types useful for therapy. This concept has been previously demonstrated in hybrid cells produced by fusing thymic lymphocytes and embryonic germ cells (Tada *et al.*, 1997).

The invention will now be exemplified by the following examples which are not intended to limit the scope of the invention as would be appreciated by a person skilled in the art.

Example 1. Effect of follicular donor cells synchronised in either G0 or G1 on *in vitro* development following nuclear transfer.

In this particular experiment, a primary cell line of follicular granulosa cells were used for nuclear transfer. The cell line was denoted as "J1" and was derived from a New Zealand Jersey heifer. J1 cells were used in this experiment between passage numbers 7-8 of culture.

Donor cells were synchronised at two stages of the cell cycle for comparison:

(1) **G0 cells** were obtained following serum deprivation by culture in medium containing 0.5% FCS for 10-11 days.

(2) **G1 cells** were fused to cytoplasts within 1-3 hours following completion of mitosis.

Negative BrdU labeling confirmed that J1 cells fused within 3 hours of mitosis had not entered S-phase and were in G1 of the cell cycle.

Embryos reconstructed with donor cells from the two cell cycle treatment groups in this experiment were cultured *in vitro* in AgR SOF medium supplemented with Life Technologies BSA (Life Technologies product number 30036-578).

Results of *in vitro* development are presented in Table1. Donor cells in G0 (control) and G1 stage of the cell cycle were equally efficient at fusing with the cytoplast (recipient cell) by means of electrically-mediated fusion. There was also no difference in the proportion of fused reconstructed embryos placed into *in vitro* culture that

developed to blastocysts between the G0 and G1 donor cell treatment groups as shown in Table 1, below.

Table 1: *In vitro* development of cloned embryos reconstructed with J1 follicular cells at either G0 or G1 stages of the cell cycle and cultured in AgR SOF medium supplemented with Life Technologies BSA (mean ± s.e.m.).

Stage of cell cycle		*Fusion*	*Grade 1-2 Blastocysts*	*Total Development*
G0	**(n=152)**	**86 ± 5.2%**	**24 ± 1.1%**	**77 ± 6.4%**
G1	**(n=186)**	**78 ± 5.0%**	**25 ± 6.2%**	**77 ± 6.9%**

Example 2. Effect of follicular donor cells (J1 and EFC cell lines) synchronised in either G0 or G1 on *in vitro* development following nuclear transfer and cultured in medium supplemented with Sigma BSA.

Further evidence is provided in Table 2 to support the finding that there is no effect of stage of cell cycle between G0 and G1 follicular donor cells on *in vitro* development of cloned embryos to the blastocyst stage following 7 days culture.

Experiments were performed with two independent follicular cell lines:

(1) "EFC"; a follicular cell line from a New Zealand Friesian dairy cow and
(2) "J1" a follicular cell line from a New Zealand Jersey dairy heifer.

EFC cells were only used in G0 while J1 cells were only used in G1 of the cell cycle in these experiments. However, the data has been combined in this example since both cell lines are derived from follicular cells and the reconstructed embryos were all cultured in the same media formulation. In these experiments, this was the standard

AgR SOF medium, but supplemented with Sigma BSA (Sigma Chemical Company; product number A-7030) rather than Life Technologies BSA, as in example 1 above.

J1 cells were used for nuclear transfer between passages 3-6, with donor cells fused within 1-3 hours following the completion of mitosis. EFC cells were used between passages 3-8 of culture and were synchronised in a G0 stage by culture in medium containing 0.5% serum for between 9-18 days.

The *in vitro* development results, presented in Table 2, show that with follicular donor cells there is no difference in the proportion of fused embryos developing to blastocysts between G0 and G1 cell cycle stages.

Table 2. *In vitro* development of cloned embryos reconstructed with follicular cells (J1 or EFC) at either G0 or G1 of the cell cycle and cultured in AgR SOF medium supplemented with Sigma BSA (mean ± s.e.m.).

Cell line	*Stage of cell cycle*		*Fusion*	*Grade 1-2 Blastocysts*	*Total Development*
J1	**G1**	**(n=576)**	**82 ± 3.8%**	**38 ± 3.6%**	**67 ± 1.8%**
EFC	**G0**	**(n=1108)**	**78 ± 2.3%**	**37 ± 2.3%**	**59 ± 3.3%**

Example 3. Effect of follicular donor cells (J1 and EFC cell lines) synchronised in either G0 or G1 on *in vivo* development following nuclear transfer.

Data is presented in Figure 1 that shows the survival throughout gestation, following the transfer to recipient cows, of cloned bovine embryos reconstructed with follicular cells either in G0 or G1 of the cell cycle. The data in Figure 1 has been complied from embryos that were produced from experiments illustrated in examples 1 and 2 above. This includes cloned embryos generated from two follicular cell lines, namely J1 and

EFC. Additionally, it includes embryos that have been produced in the same AgR SOF media formulation but supplemented with either Sigma BSA or Life Technologies BSA. The data have been pooled since there is no effect on post-transfer viability between either of these two follicular cell lines nor an effect of the two sources of BSA (although there is a significant effect on development to the blastocyst stage).

The data in Figure 1 represents a total of 85 embryos reconstructed with G0 donor cells and 95 embryos reconstructed with early G1 donor cells that were transferred to the reproductive tracts of synchronised recipient cows.

Figure 1 plots the percentage of embryos / fetuses present throughout gestation, from day 7 embryo transfer to term, as determined by regular ultrasonography, rectal palpation and calving. Most notably, the use of G1 follicular cells resulted in the birth of viable calves at full term. Compared to G0 donor cells, there was a tendency for embryonic survival with cloned embryos reconstructed with G1 cells to be lower from day 30 of gestation right through to full term, however, this did not reach a level of statistical significance with the numbers of transfers reported here.

With G1 follicular cells, 9% of embryos transferred (9/95) resulted in the birth of calves at full term. However, 4 of these calves died at or shortly after birth resulting in an overall efficiency of 5% viable cloned calves from G1 cells (5/95). In comparison, G0 donor follicular cells resulted in 20% development to full term (17/85), with 3 calves dying during birth culminating in an overall 16% efficiency of viable calves produced (14/85).

Example 4. Effect of adult female skin fibroblasts (Age + and Age - cell lines) synchronised in either G0 or G1 on *in vitro* and *in vivo* development following nuclear transfer and culture in medium supplemented with Life Technologies BSA.

Data have been combined from experiments with two independent adult skin fibroblast primary cell lines. The two cell lines are denoted "Age +" and "Age –". They are both female cell lines from Angus beef cows selected for either late or early onset to puberty, respectively. As there were no significance differences in terms of the *in vitro* and *in vivo* development between these two similar cell lines, the data have been combined in Table 3 and Figure 2 below.

Two stages of the cell cycle were compared in these experiments:

(1) **G1**, whereby donor cells were fused to cytoplasts within 1-3 hours post completion of mitosis.

(2) **G0**, whereby donor cells where cultured for 3-12 days in medium supplemented with 0.5% FCS. For those reconstructed embryos that were transferred to recipient cows, cells were deprived of serum for 4-5 days.

Cells from both cell lines and both cell cycle treatments were used for nuclear transfer at passage 7 of culture. Reconstructed embryos where cultured in the standard AgR SOF media formulation, supplemented with Life Technologies BSA (Life Technologies product number 30036-578).

The data presented in Table 3 show an effect of length of time G0 cells were in low serum on fusion efficiency, however, once fused with the cytoplast there was no effect on subsequent *in vitro* development and so, the G0 blastocyst data have been combined. There was no effect of cell cycle stage between G0 and G1 on development to blastocyst stages.

Table 3. *In vitro* development of cloned bovine embryos reconstructed with adult female skin fibroblast cells (Age + and Age -) at either G0 or G1 of the cell cycle and cultured in AgR SOF medium supplemented with Life Technologies BSA (mean ± s.e.m.).

Stage of cell cycle		*Fusion*	*Grade 1-2 Blastocysts*	*Total Development*
G0	**3-5 days (n=411)**	**66 ± 1.9%[a]**		
			19 ± 3.0%	**58 ± 4.5%**
	7-12 days (n=107)	**41 ± 6.9%[b]**		
G1	**(n=401)**	**59 ± 2.5%[ab]**	**19 ± 5.0%**	**67 ± 6.6%**

ab $P<0.05$

The data in Figure 2 demonstrate that adult skin fibroblasts selected in G1 are capable of producing viable calves at full term. Similar to the data in Figure 1, there is a tendency for survival at full term to be greater with G0, however, from the numbers of transfers performed here this is not statistically significant. With G1 donor cells, 4% of embryos transferred yielded viable cloned calves at term (1/25). In comparison, G0 donor cells resulted in 14% viable development to term (3/22). In this experiment, all 4 calves delivered survived the post-natal period.

Example 5. Effect of adult skin fibroblasts (LJ801 and 3XTC cell lines) synchronised in either G0 or G1 on *in vitro* and *in vivo* development following nuclear transfer and culture in medium supplemented with ICP BSA.

Additional experiments comparing G0 and G1 cell cycle stages with two independent adult skin fibroblast cell lines were conducted. The two cell lines were denoted "LJ801" and "3XTC". LJ801 is a male cell line derived from a Limousine X Jersey

steer, while 3XTC is derived from a crossbred cow which on three occasions has delivered triplet calves.

Two cell cycle stages were compared:

(1) **G0 cells**, whereby donor cells in both cell lines were cultured in medium with 0.5% FCS for 4-5 days; and

(2) **G1 cells**, whereby donor cells were fused to cytoplasts within 1-3 hours following the completion of mitosis.

Negative BrdU labeling confirmed that adult skin fibroblast cells fused within 3 hours of mitosis had not entered S-phase.

Both LJ801 and 3XTC were used for nuclear transfer experiments between passages 3-4 of culture.

The data in Table 4 have been combined since there was no difference in the efficiency of development to blastocyst stages between LJ801 and 3XTC cells and reconstructed embryos from both cell lines were cultured in the same standard AgR SOF media but supplemented with an alternative BSA source, this time from ICP (Immuno-Chemical Products, Auckland, New Zealand; product number ABFF-002). The data on embryonic survival throughout gestation, following embryo transfer, is presented in Figures 3 and 4 in terms of the respective cell lines 3XTC and LJ801.

The data in Table 4 show that there was no effect of the cell cycle of donor adult skin fibroblasts synchronised in either G0 or G1 on development to the blastocyst stage.

Table 4. *In vitro* development of cloned embryos reconstructed with adult skin fibroblast cells (LJ801 and 3XTC cells) at either G0 or G1 of the cell cycle and cultured in AgR SOF medium supplemented with ICP BSA (mean ± s.e.m.).

Stage of cell cycle		*Fusion*	*Grade 1-2 Blastocysts*	*Total Development*
G0	**(n=145)**	**72 ± 7.8%**	**36 ± 6.1%**	**61 ± 5.0%**
G1	**(n=200)**	**60 ± 3.1%**	**37 ± 6.0%**	**57 ± 5.0%**

The data in Figure 3 show that with embryos reconstructed with 3XTC cells that there was no difference in survival to day 150, at least. With donor cells in G1 of the cell cycle embryonic survival at day 150 was 25% (3/12) compared to 27% for G0 donor cells (3/11).

The data in Figure 4 show that with embryos reconstructed with LJ801 fibroblast cells that whilst there is a trend for embryonic survival to be greater at day 210 of gestation with G0 donor cells, this is not statistically significant. With donor cells in G1 of the cell cycle embryonic survival at day 210 was 23% (3/13) compared to 39% for G0 donor cells (7/18).

Example 6. Effect of genetically modified bovine fetal fibroblast cells synchronised in either G0 or G1 on *in vitro* and *in vivo* development following nuclear transfer.

An experiment was also performed with genetically modified bovine fetal female lung fibroblast cells to investigate the effect of cell cycle stage on development following nuclear transfer. The genetic modification involved the random insertion of additional

copies of bovine β and κ-casein genes. The transgenic cell line was denoted "casein plus 5110".

Two cell cycle stages were compared:

(1) **G0 cells**, whereby donor cells were cultured in medium with 0.5% FCS for 3-6 days; and

(2) **G1 cells**, whereby donor cells were fused to cytoplasts within 1-3 hours following the completion of mitosis.

Reconstructed embryos were cultured in the standard AgR SOF media formulation, supplemented with ICP BSA (Immuno-Chemical Products, Auckland, New Zealand; product number ABFF-002).

The data on *in vitro* embryo development is presented in Table 5. Development to blastocyst stages with the casein plus 5110 cells was significantly less when donor cells used for nuclear transfer were in G1 compared to G0. This result is in contrast to the other cell lines presented in the previous examples above.

Table 5. *In vitro* development of cloned cattle embryos reconstructed with transgenic female fetal lung fibroblast cells (casein plus 5110 cells) at either G0 or G1 stages of the cell cycle and cultured in AgR SOF medium supplemented with ICP BSA (mean ± s.e.m.).

Stage of cell cycle		*Fusion*	*Grade 1-2 Blastocysts*	*Total Development*
G0	**(n=150)**	**90 ± 8.1%**	**52 ± 1.8%[a]**	**72 ± 2.9%[c]**
G1	**(n=73)**	**77 ± 6.5%**	**26 ± 5.1%[b]**	**43 ± 2.2%[d]**

ab P<0.05; cd P<0.01

The embryonic survival data presented in Figure 5 shows no effect of stage of cell cycle or development to day 90 at least, with casein plus 5110 transgenic cells. With donor cells in G1 of the cell cycle embryonic survival at day 90 was 38% (9/24) compared to 27% for G0 donor cells (6/22).

Example 7. Effect of non-proliferating, senescent donor cells (in late G1 phase) on in vitro and in vivo development following nuclear transfer.

A nuclear transfer experiment was performed to investigate the effect on development with non-proliferating, senescent bovine donor cells. The cells used in this experiment were fetal female lung fibroblasts and had been genetically modified (denoted as "561 cells"). Initially, the cells were actively growing, however, during the course of their culture this progressively slowed and at late passage the cells entered a non-proliferative phase, known by those skilled in the art as senescence, whereby cell division ceases. Senescent cells are known to arrest in G1 of the cell cycle, specifically at the late G1 / S-phase boundary (Sherwood et al., 1988). When cells enter senescence they block at late G1 and they fail to enter S-phase in response to physiological mitogens. Thus, senescence is distinct from quiescence, whereby in the latter situation cells may be induced to re-enter the cell cycle and proliferate upon return to optimal conditions (eg addition of serum in the case of serum deprived cell cultures).

Embryos reconstructed with transgenic senescent cells were cultured in the AgR SOF media, supplemented with ICP BSA.

The data on in vitro embryo development is presented in Table 6. Development to blastocyst stages with the senescent donor cells was lower than that expected for G0 or early G1 donor cells as illustrated in the previous examples.

Table 5. In vitro development of cloned cattle embryos reconstructed with non-proliferating, senescent transgenic fibroblasts (561 cells) and cultured in AgR SOF medium supplemented with ICP BSA.

Cell line	Stage of cell cycle	Fusion	Grade 1-2 Blastocysts	Total Development
561	Senescent (n=158)	88%	12%	35%

The embryonic survival data presented in Figure 6 suggests a low efficiency of cloning with non-proliferating, senescent cells arrested in late G1 phase, with only 4% of embryos developing to day 90 (1/26).

CONCLUSIONS

1. It is possible to select individual cells at a definitive stage of the cell cycle, preferably early G1. This is advantageous compared to prior art using so-called proliferating cells where the actual stages of the cell cycle are not accurately known for each individual cell used for nuclear transfer. The same is true for serum starved populations of cells.

2. Post-mitotic cells in the early G1 phase of the cell cycle are totipotent following nuclear transfer as evidenced by the production of viable calves.

3. Thus, G0 is not the only stage of the cell cycle that is compatible with development following nuclear transfer with differentiated cultured cells and G1, preferably early G1, nuclei can also be functionally reprogrammed.

4. Post-mitotic early G1 cells promote development to the blastocyst stage following nuclear transfer to similar levels as cells in G0.

5. There is no significant difference in the post-transfer viability to full term of cloned embryos reconstructed with either G0 or early G1 donor nuclei.

INDUSTRIAL APPLICATION

The present invention may be useful in establishing cloned herds/flocks of animals including transgenic animals capable of producing pharmaceutically useful proteins, agriculturally useful products such as meat, milk and fibre, as well as human applications especially in the field of therapeutic cloning.

It will be appreciated that the description is not intended to limit the scope of the invention to the above examples only, many variations such as might readily occur to a person skilled in the art being possible, without departing from the scope of the appended claims.

REFERENCES

Barnes F L; Collas P; Powell R; King W A; Westhusin M and Shepherd D (1993). Influence of recipient oocyte cell cycle stage on DNA synthesis, nuclear envelope breakdown, chromosome constitution and development in nuclear transplant bovine embryos. *Molecular Reproduction and Development* 36: 33-41.

Boquest A C; Day B N and Prather R S (1999). Flow cytometric cell cycle analysis of cultured porcine fetal fibroblast cells. *Biology of Reproduction* 60: 1013-1019.

Campbell K H S; Loi P; Cappai P and Wilmut I (1994). Improved development to blastocyst of ovine nuclear transfer embryos reconstructed during the presumptive S-phase of enucleated activated oocytes. *Biology of Reproduction* 50: 1385-1393.

Campbell K; McWhir J; Ritchie B and Wilmut I (1995). Production of live lambs following nuclear transfer of cultured embryonic disc cells. *Theriogenology* 43: 181.

Campbell K H S; Loi P; Otaegui P J and Wilmut I (1996a). Cell cycle co-ordination in embryo cloning by nuclear transfer. *Reviews in Reproduction* 1: 40-46.

Campbell K H S; McWhire J; Ritchie W A and Wilmut I (1996b). Sheep cloned by nuclear transfer from a cultured cell line. *Nature* 380: 64-66.

Cibelli J B; Stice S L; Golueke P J; Kane J J; Jerry J; Blackwell C; Ponce De Leon F A and Robl J M (1998). Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* 280: 1256-1258.

Collas P; Balise J J and Robl J M (1992a). Influence of cell cycle stage of the donor nucleus on development of nuclear transplant rabbit embryos. *Biology of Reproduction* 46: 492-500.

Collas P; Pinto-Correia C; Ponce De Leon F A and Robl J M (1992b). Effect of donor cell cycle stage on chromatin and spindle morphology in nuclear transplant rabbit embryos. *Biology of Reproduction* 46: 501-511.

Czolowska R; Modlinski J A and Tarkowski A K (1984). Behaviour of thymocyte nuclei in non-activated and activated mouse oocytes. *Journal of Cell Science* 69: 19-34.

Gadbois D M, Crissman H A, Tobey R A and Bradbury E M (1992). Multiple kinase arrest points in the G1 phase of nontransformed mammalian cells are absent in transformed cells *Proceedings of the National Academy of Sciences, USA* 89: 8626-8630.

Gardner D K; Lane M; Spitzer A and Batt P (1994). Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: amino acids, vitamins and culturing embryos in groups stimulate development. *Biology of Reproduction* 50: 390-400.

Pedersen R A (1999). Embryonic stem cells for medicine. *Scientific American* April 1999: 44-49.

Perry ACF, Wakayama T, Kishikawa H, Kasai T, Okabe M, Toyoda Y and Yanagimachi R (1999). Mammalian transgenesis by intracytoplasmic sperm injection. *Science* 284: 1180-1183.

Schnieke A E; Kind A J; Ritchie W A; Mycock K; Scott A R; Ritchie M; Wilmut I; Colman A and Campbell KHS (1997). Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. *Science* 278: 2130-2133.

Sherwood S W, Rush D, Ellsworth J L and Schimke R T (1988). Defining cellular senescence in IMR-90 cells: a flow cytometric analysis. *Proceedings of the National Academy of Sciences, USA* 85: 9086-9090.

Stice S L; Strelchenko N S; Keefer C L and Matthews L (1996). Pluripotent bovine embryonic cell lines direct embryonic development following nuclear transfer. *Biology of Reproduction* 54: 100-110.

Susko-Parrish J L; Leibfried-Rutledge M L; Northey D L; Schutzkus V and First N L (1994). Inhibition of protein kinases after an induced calcium transient causes transition of bovine oocytes to embryonic cycles without meiotic completion. *Developmental Biology* 166: 729-739.

Tada M, Tada T, Lefebvre L, Barton S C and Surani M A (1997). Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells. *The EMBO Journal* 16: 6510-6520.

Thompson J G E; Simpson A C; Pugh PA; Donnelly P E and Tervit H R (1990). The effect of oxygen concentration on the in vitro development of preimplantation sheep and cattle embryos. *Journal of Reproduction and Fertility* 89: 573-578.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS and Jones JM (1998). Embryonic stem cell lines derived from human blastocysts. *Science* 282: 1145-1147.

Vignon X; Chesne P; Le Bourhis D; Flechon J E; Heyman Y and Renard J P (1998). Developmental potential of bovine embryos reconstructed from enucleated matured oocytes fused with cultured somatic cells. *C R Academy of Science, Paris* 321: 735-745.

Vignon X; Le Bourhis D; Chesne P; Marchal J; Heyman Y and Renard J P (1999). Development of bovine nuclear transfer embryos reconstituted with quiescent and proliferative skin fibroblasts. *Theriogenology* 51: 216.

Wakayama T and Yanagimachi R (1999). Cloning adult male mice from adult tail-tip cells. *Nature Genetics* 22: 127-128.

Wall RJ, Kerr DE and Bondioli KR (1997). Transgenic dairy cattle: Genetic engineering on a large scale. *Journal of Dairy Science* 80: 2213-2224.

Wells D N; Misica P M; McMillan W H and Tervit H R (1998). Production of cloned bovine fetuses following nuclear transfer with cells from a fetal fibroblast cell line. *Theriogenology* 49: 330.

Wells D N; Misica P M; Tervit H R and Vivanco W H (1999). Adult somatic cell nuclear transfer is used to preserve the last surviving cow of the Enderby Island cattle breed. *Reproduction, Fertility and Development* 10: 369-378.

Wilmut I, Schnieke A E; McWhir J; Kind A J and Campbell K H S (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature* 385: 810-813.

Zakhartchenko V; Durchova-Hills G; Stojkovic M; Schernthaner W; Prelle K; SteinbornR; Muller M; Brem G and Wolf E (1999). Effects of serum starvation and re-cloning on the efficiency of nuclear transfer using bovine fetal fibroblasts. *Journal of Reproduction and Fertility* 115: 325-331.

All references are incorporated herein in their entirety by reference.

CLAIMS

1. A method of nuclear transfer, comprising selecting and segregating G1 cells from a proliferating or non-proliferating population of diploid donor cells and transferring a nucleus from such a segregated G1 cell into an enucleated recipient cell, wherein donor cells are selected and segregated by physical picking based on individual cell identification to produce a pure G1 cell population with the proviso that said diploid donor cells are not selected from blastomeres which have been synchronised by ≥5μM nocodazole or 5 μg/ml colcemid.

2. A method as claimed in claim 1, wherein the donor cell population is at one or more known or unknown stages of the cell cycle.

3. A method as claimed in claim 1 or 2, wherein said donor cell population is non-proliferating and has been synchronised at any point in the G1 stage of the cell cycle.

4. A method as claimed in any one of claims 1 to 3 wherein said G1 cell is segregated at an early G1 phase.

5. A method as claimed in any one of claims 1 to 3, wherein the donor cell population is non-proliferating and comprises senescent cells.

6. A method as claimed in any one of claims 1 to 5, wherein said donor cell population is derived from either embryo, fetal, juvenile or adult cells isolated from an animal *in vivo* or from a cell culture *in vitro*.

7. A method as claimed in claim 6, wherein said donor cell population comprises any diploid karyotypically normal cell capable of being stimulated to enter the cell cycle and proliferate.

8. A method as claimed in claim 7, wherein said donor cell population is of an undifferentiated cellular state or are at any degree of differentiation or quiescence or senescence.

9. A method as claimed in any preceding claim wherein the donor cells are adult or fetal fibroblasts or follicular cells.

10. A method as claimed in any preceding claim wherein said donor cells comprise genetically modified cells.

11. A method as claimed in claim 10 wherein said donor cells comprise transgenic cells.

12. A method as claimed in any preceding claim, wherein the recipient cell comprises an enucleated oocyte.

13. A method as claimed in claim 12, wherein the enucleated oocyte is obtained from a species corresponding in origin to the donor nuclei.

14. A method as claimed in any one of claims 1 to 11, wherein the recipient cell comprises an enucleated stem cell or a clump of enucleated stem cells fused together.

15. A method as claimed in claim 14, wherein the stem cells are embryonic stem cells isolated from a growing embryo or form an established cell line in culture.

16. A method of producing cloned animal embryos which comprises transferring a diploid donor nucleus from a cell selected and segregated in the G1 stage of the cell cycle according to claim 1 into an enucleated recipient cell, with the proviso that said diploid donor cells are not selected from blastomeres which have been synchronised at G1-phase by $\geq 5\mu M$ nocodazole or 5 µg/ml colcemid.

17. A method as claimed in claim 16, wherein the donor nuclei are genetically altered using methods well known in the art to produce cloned embryos having desirable genetic traits.

18. A method as claimed in claim 16 or 17, when used to produce an animal species of cloned embryo selected from the group comprising birds, amphibia, fish and mammals.

19. A method as claimed in claim 18, wherein said cloned animal embryo is a mammal, selected from the group comprising primates including humans, rodents, rabbits, cats, dogs, horses, cattle, sheep, deer, goats and pigs.

20. A reconstituted animal embryo prepared by the method claimed in claim 16.

21. A reconstituted animal embryo as claimed in claim 17, comprising a transgenic embryo.

22. A reconstituted animal embryo as claimed in claim 20 or 21 re-cloned to further increase embryo numbers or which undergoes serial nuclear transfer to aid nuclear reprogramming and/or development.

23. A reconstituted animal embryo as claimed in any one of claims 20 to 22, comprising a species of mammal selected from the group comprising primates including humans, rodents, rabbits, cats, dogs, horses, cattle, sheep, deer, goats and pigs.

24. A method of cloning a non-human animal comprising the steps: (1) producing a cloned non-human animal embryo according to the method of any one of claim 16 or 17 (2) allowing a non-human animal to develop to term from the embryo by known methods; and (3) optionally breeding from the non-human animal so formed either by conventional methods or by further cloning.

25. A method as claimed in claim 24, wherein said cloned non-human animal is a non-human mammal selected from the group comprising non-human primates, rodents, rabbits, cats, dogs, horses, cattle, sheep, and deer.

26. A method as claimed in claim 24 or 25, wherein said cloned non-human animal is a transgenic non-human animal having a desirable genetic trait.

27. A method as claimed in claim 26, wherein said transgenic non-human animal is a transgenic bovine or ovine.

28. A cloned non-human animal prepared by the method of claim 24.

29. A cloned non-human animal as claimed in claim 28 comprising a mammal selected from the group comprising non-human primates, rodents, rabbits, cats, dogs, horses, cattle, sheep, and deer.

30. A cloned non-human animal as claimed in claim 28 or 29 comprising a transgenic non-human animal having a desirable genetic trait.

31. A cloned non-human animal as claimed in claim 30 comprising a transgenic bovine or ovine.

32. A cloned transgenic animal as claimed in claim 30 or 31 wherein the desirable genetic trait is selected from the insertion, deletion, or modification of a gene or genes enabling the production of pharmaceutical proteins in milk, blood or urine; production of nutraceutical products in milk or meat; production of beneficial agricultural traits to improve the quality of milk, meat and fibre production; improve resistance to pests and diseases; production of industrial proteins in milk; xenotransplantation; and for the generation of transgenic animals as models for human disease.

33. Offspring and descendants of the cloned non-human animal as claimed in any one of claims 28 to 32.

ART 34 AMDT

34. A method of producing a cell line comprising the steps a) selecting and segregating G1 cells from a proliferating population of diploid donor cells or from a synchronised population of diploid G1 cells or from a population of diploid senescent cells, and transferring a nucleus from such a segregated cell into an enucleated recipient cell; b) optionally growing to embryo stage; c) recovering cells; and d) establishing an immortalised cell line *in vitro* by methods known in the art.

35. A method as claimed in claim 34, wherein said cell line is an embryonic stem cell line.

36. A method as claimed in claim 34 or 35, wherein said donor cells are human cells.

37. A method as claimed in any one of claims 34 to 36, wherein both donor and recipient cells are human cells.

38. A method as claimed in any one of claims 34 to 37, wherein the donor cells are adult or fetal cells selected from any karyotypically normal cell type and the recipient cells are selected from any cell type capable of reprogramming gene expression.

39. An embryonic cell line produced by the method of any one of claims 34 to 36.

40. A human embryonic stem cell line produced by the method of claim 36, when dependent upon claim 35, useful in therapeutic applications.

41. A method of producing stem cells comprising the steps of a) selecting and segregating G1 cells from a proliferating population of diploid donor cells or from synchronised population of diploid G1 cells or from a population of diploid senescent cells and transferring a nucleus from such a segregated cell into an enucleated recipient cell; b) optionally growing to embryo stage; and c) recovering stem cells.

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42. A method as claimed in claim 41, wherein said stem cells are embryonic stem cells.

43. A method as claimed in claim 41 or 42, wherein said donor cells are human cells.

44. A method as claimed in any one of claims 41 to 43, wherein both donor and recipient cells are human cells.

45. A method as claimed in any one of claims 41 to 44, wherein the donor cells are adult or fetal cells selected from any karyotypically normal cell type and the recipient cells are selected from any cell type capable of reprogramming gene expression.

46. Embryonic stem cells produced by the method of any one of claims 42 to 44.

47. Embryonic stem cells as claimed in claim 46, comprising human embryonic stem cells.

48. A use of the embryonic cells of any one of claims 39, 40 and 46, wherein specialised types of cell or tissue selected from the group comprising nerve cells, muscle cells, heart cells, liver cells, lung cells, kidney cells or any other type of cell of interest are cultured using methods well known in the art.

49. A use as claimed in claim 48, wherein said embryonic cells are human embryonic stem cells as claimed in claim 40 or 47.

50. A method of therapeutic cloning, wherein embryonic stem cells are produced according to any one of claims 35 and 41 to 44 from a donor cell derived from a subject, and cultured to produce specialised cells or tissue for transplantation in said subject or in another subject in need of such treatment.

51. A method as claimed in claim 50, wherein said embryonic stem cells comprise one or more transgenes to confer a desirable genetic trait in the resulting differentiated cells used for transplantation.

52. A method of treating a disease, disorder or injury which may be treated by transplantation of specialised cells or tissue, comprising administering to a patient in need thereof a therapeutically effective amount of specialised cells or tissue produced according to the method of claim 50 or 51.

53. A method as claimed in claim 50 or 51, wherein said disease, disorder or injury is selected from various neurological disorders (*eg* Parkinson's disease), diabetes, heart disease, muscular dystrophy, various hereditary diseases, specific cancers (*eg* leukemia), spinal cord injury, burns and other afflictions.

54. A method of drug discovery or toxicology testing of drugs using *in vitro* differentiated human embryonic stem cells produced by the methods of claim 48.

55. A method of xenotransplantation, wherein cells, tissues and organs are isolated from the non-human cloned animal of any one of claims 28 to 32, and used for transplantation in a human patient in need thereof.

56. A method of gene therapy, wherein cells, tissues and organs comprise a transgene and are isolated for the non-human cloned animal of claim 30 or 31.

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Published:
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(54) Title: NUCLEAR TRANSFER WITH SELECTED DONOR CELLS

(57) Abstract: The present invention provides a method of nuclear transfer by selecting and segregating G1 cells from a donor cell population. This method is advantageous over the prior art as it provides certainty as to the stage of the cell cycle which the donor nuclei are in and allows for the production of cloned transgenic or non-transgenic embryonic cells, reconstituted embryos and whole animals for agricultural, pharmaceutical, nutraceutical and biomedical applications.

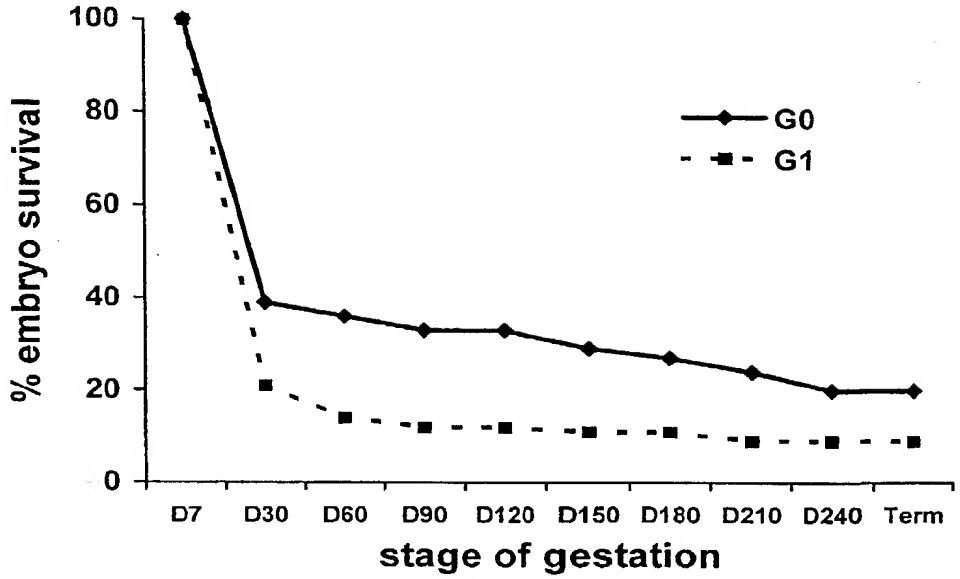

FIGURE 1

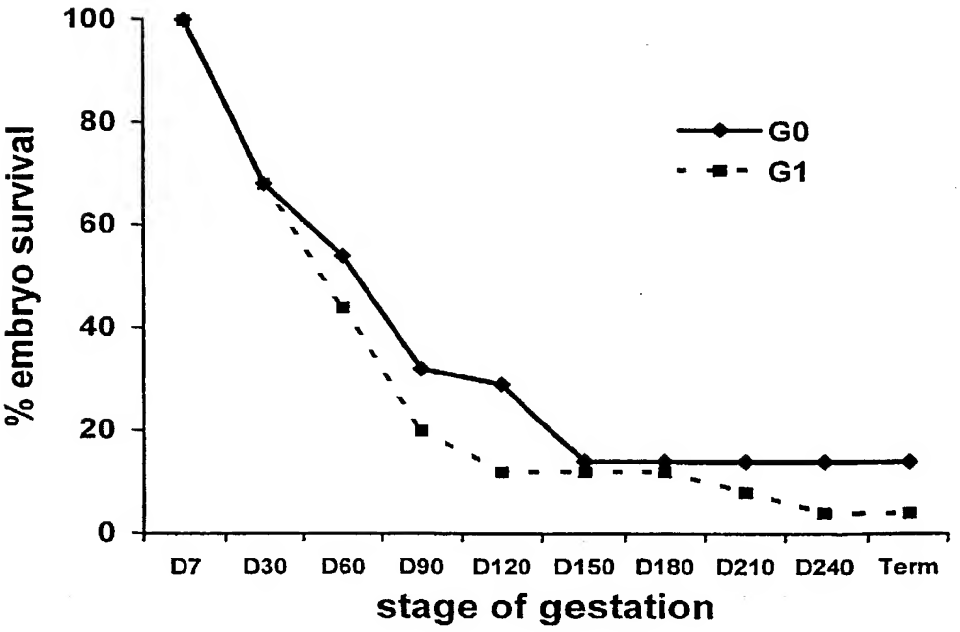

FIGURE 2

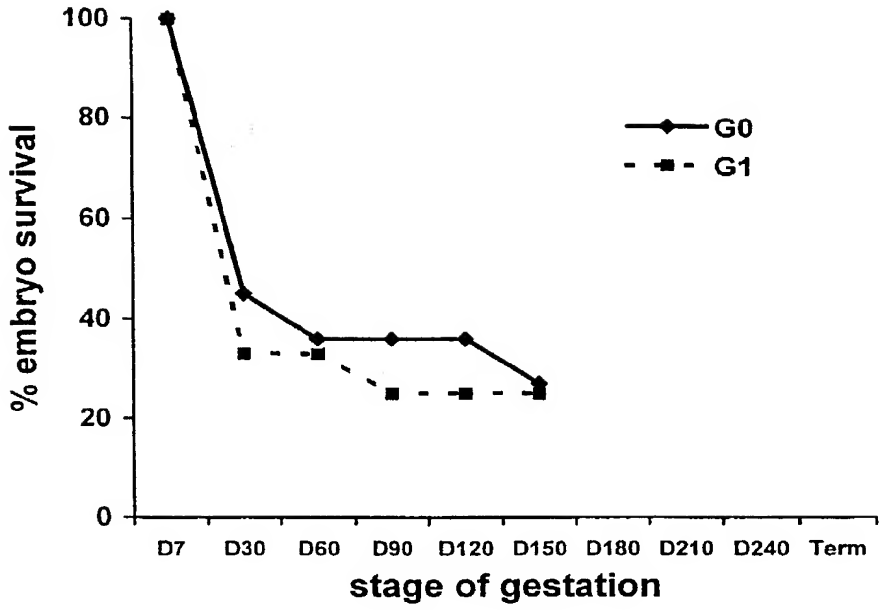

FIGURE 3

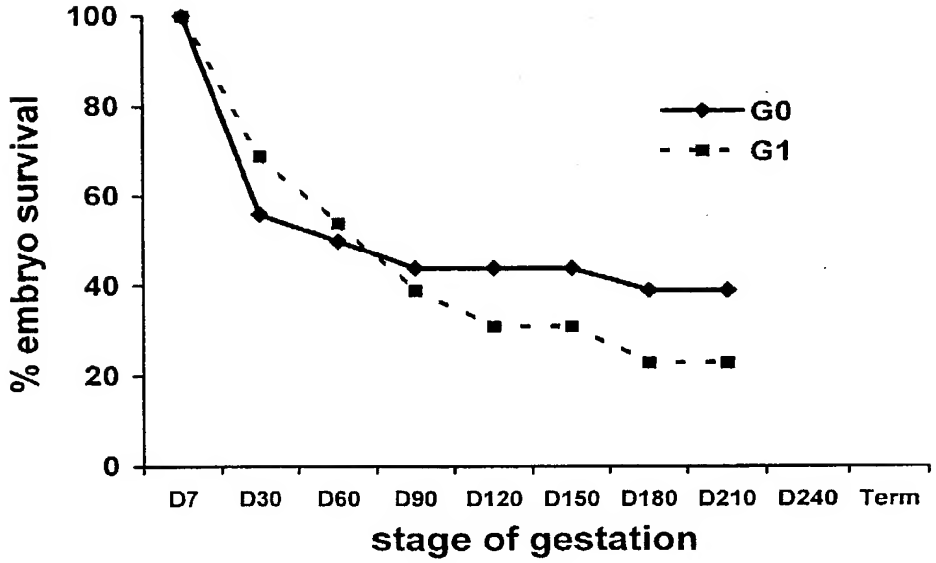

FIGURE 4

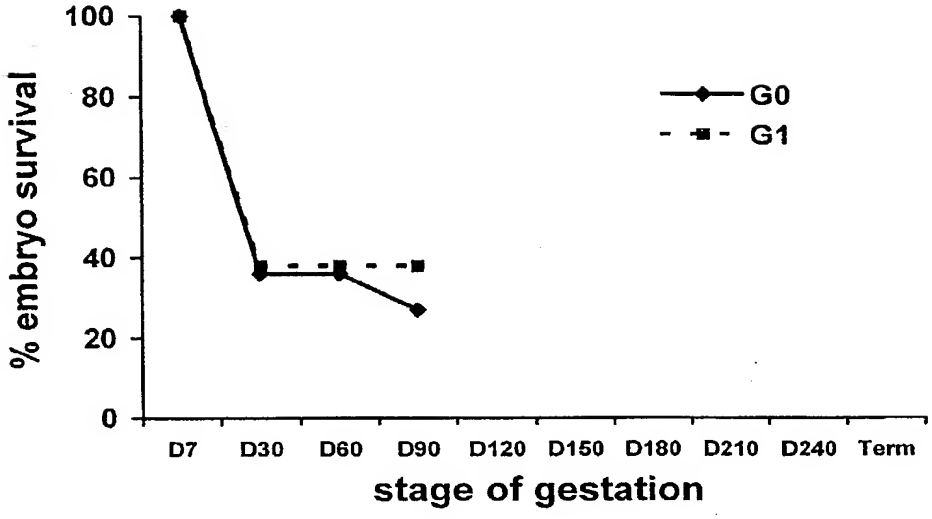

FIGURE 5

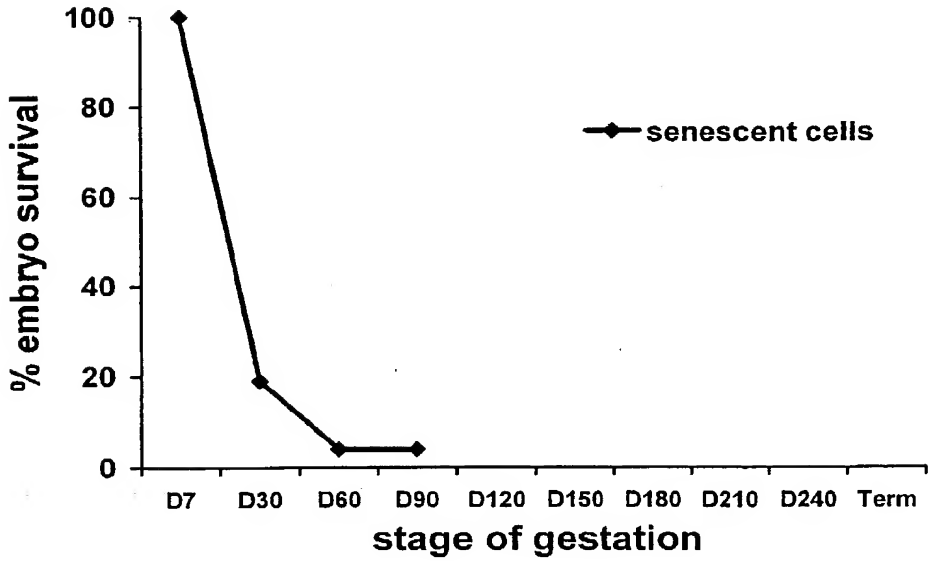

FIGURE 6

43

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: David Wells	§	
	§	
	§	Group Art Unit: Unknown
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	§	Examiner: Unknown
Filed: March 14, 2002	§	
	§	Atty. Dkt.: 4070.000300
For: NUCLEAR TRANSFER WITH SELECTED DONOR CELLS	§	
	§	

ELECTION UNDER 37 C.F.R. §§ 3.71 AND 3.73 AND POWER OF ATTORNEY

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

The undersigned, being Assignee of record of the entire interest in the above-identified application by virtue of an assignment recorded in the United States Patent and Trademark Office as set forth below, hereby elects, under 37 C.F.R. § 3.71, to prosecute the application to the exclusion of the inventor.

The Assignee hereby revokes any previous Powers of Attorney and appoints:

Danny L. Williams, Reg. No. 31,892; Terry D. Morgan, Reg. No. 31,181; J. Mike Amerson, Reg. No. 35,426; Kenneth D. Goodman, Reg. No. 30,460; Jeffrey A. Pyle, Reg. No. 34,904; Jaison C. John, Reg. No. 50,737; Ruben S. Bains, Reg. No. 46,532; Steven Koon Hon Wong, Reg. No. 48,459; Scott F. Diring, Reg. No. 35,119; George J. Oehling, Reg. No. 40,471; Shelley P.M. Fussey, Reg. No. 39,458; Mark D. Moore, Reg. No. 42,903; Louis H. Iselin, Reg. No. 42,684; Raymund F. Eich, Reg. No. 42,508; Daren C. Davis, Reg. No. 38,425; and Stephanie A. Wardwell, Reg. No. 48,025;

each an attorney or agent of the firm of WILLIAMS, MORGAN & AMERSON, P.C., as its attorney or agent for so long as they remain with such firm, with full power of substitution and revocation, to prosecute the application, to make alterations and amendments therein, to transact all business in the Patent and Trademark Office in connection therewith, and to receive any Letters Patent, and for one year after issuance of such Letters Patent to file any request for a certificate of correction that may be deemed appropriate.

Pursuant to 37 C.F.R. § 3.73, the undersigned has reviewed the evidentiary documents, specifically the Assignment to AgResearch Limited, referenced below, and certifies that to the best of my knowledge and belief, title remains in the name of the Assignee.

Please direct all communications as follows:

Shelley P.M. Fussey
WILLIAMS, MORGAN & AMERSON, P.C.
7676 Hillmont, Suite 250
Houston, Texas 77040
(713) 934-7000

ASSIGNEE:
AgResearch Limited

By: Ian K. Boddy
Name: Ian Kenneth Boddy
Title: Commercial Manager

Date: 09/05/02

ASSIGNMENT:

- ☒ Concurrently filed
- ☐ Previously recorded
  - Date: _____
  - Reel: _____
  - Frames: _____

PATENT
4070.000300

DECLARATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or the below named inventors are the original, first and joint inventors (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **Nuclear Transfer With Selected Donor Cells**, the Specification of which:

☐ is attached hereto.
☒ was filed on **March 14, 2002** as Application Serial No. **10/088,129**.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims.

I acknowledge the duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability of the subject matter claimed in this application, as "materiality" is defined in Title 37, Code of Federal Regulations, § 1.56.

I hereby claim priority benefits under Title 35, United States Code, § 119 and/or § 365 of any foreign application(s) for patent or inventor's certificate, PCT international application(s), and United States provisional application(s), listed below and have also identified below any foreign application for patent or inventor's certificate, PCT international application, or United States provisional application, having a filing date before that of the application on which priority is claimed:

PRIORITY APPLICATION(S)			**Priority Claimed**
PCT/NZ00/00179	PCT	September 14, 2000	Yes
(Number)	(Country)	(Date Filed)	Yes/No
337792	NZ	September 14, 1999	Yes
(Number)	(Country)	(Date Filed)	Yes/No

I hereby claim the benefit under Title 35, United States Code, § 120 and/or § 365 of any United States application(s) and PCT international application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose all information known to me to be material to patentability of the subject matter claimed in this application, as "materiality" is defined in Title 37, Code of Federal Regulations, § 1.56, which becomes available between the filing date of the prior application and the national or PCT international filing date of this application:

N/A		
(Application Serial No.)	(Filing Date)	(Status)
(Application Serial No.)	(Filing Date)	(Status)

I hereby direct that all correspondence and telephone calls be addressed to Shelley P.M. Fussey, Williams, Morgan & Amerson, P.C., 7676 Hillmont, Suite 250, Houston, Texas 77040, (713) 934-7000.

I hereby declare that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

Inventor's Full Name:	~~David~~	Norman	Wells
Inventor's Signature:	D. N. Wells		
Country of Citizenship:	New Zealand	Date: 9/5/02	
Residence Address: (street, number, city, state, and/or country)	52 Enderley Avenue Hamilton, New Zealand NZX		
Post Office Address: (if different from above)			